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ELEMENTS

OF

WATER BACTERIOLOGY

WITH SPECIAL REFERENCE TO

SANITARY WATER ANALYSIS

BY

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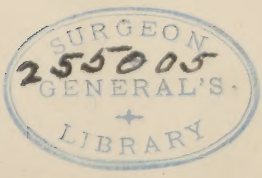

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TO

WILLIAM THOMPSON SEDGWICK

BY TWO OF HIS PUPILS

AS A TOKEN OF GRATEFUL AFFECTION

PREFACE TO FOURTH EDITION

Since the first edition of this book appeared, nearly twenty years ago, it has naturally been subject to frequent changes with the progress of the branch of applied science with which it deals. New editions appeared in 1908 and in 1915 and the passage of eight years has now necessitated a more radical revision than any of those previously undertaken.

The more important changes which have taken place are concerned with the increased definiteness and precision in the use of the colon test. The progress of the years has made it possible to omit much historical matter now out of date and to concentrate upon the procedures which have proved themselves of definite value. We have therefore materially condensed our discussion of this subject, Chapters V and VI of the present edition corresponding to Chapters VI, VII, and VIII of the earlier volumes, and at the same time have sought to incorporate new matter including the latest recommendations of the Committee on Standard Methods of the American Public Health Association, which appeared only a few weeks ago.

Similar considerations have led us to reduce materially the amount of space devoted to the isolation of the typhoid bacillus, since this step is so rarely undertaken by practical water bacteriologists, and to combine the brief discussion of this subject which seemed necessary with the discussion of other intestinal bacteria in Chapter VII of the present edition.

Throughout the volume we have attempted to bring all the subjects discussed up to date and to omit the less important details in historical development while at the same time preserving a general background so that the student may understand the lines along which water bacteriology has grown.

In all respects we have accepted the procedures outlined in the last report of the Committee on Standard Methods but we have assumed that a copy of the report of the committee would be in every water laboratory, and have therefore omitted in the present edition detailed methods for the preparation of media. The

standard methods report is an essential part of the equipment of every water laboratory, but we believe that a critical discussion of the wider principles involved, such as is presented in this volume, will still prove of value to the water bacteriologist and will be essential to the student who desires to acquire a fundamental comprehension of the problem with which he is to deal.

We have retained the complete list of references published at the end of the earlier editions and have brought it up to date (June, 1923) feeling that a reasonably complete bibliography of the subject of water bacteriology would prove of substantial and continuing value.

PREFACE TO FIRST EDITION

The general awakening of the community to the importance of the arts of sanitation — accelerated by the rapid growth of cities and the new problems of urban life — demands new and accurate methods for the study of the microbic world. Bacteriology has long since ceased to be a subject of interest and importance to the medical profession merely, but has become intimately connected with the work of the chemist, the biologist and the engineer. To the sanitary engineer and the public hygienist a knowledge of bacteriology is indispensable.

In the swift development of this science during the last ten years perhaps no branch of bacteriology has made more notable progress than that which relates to the sanitary examination of water. After a brief period of extravagant anticipation, and an equally unreasonable era of neglect and suspicion, the methods of the practical water bacteriologist have gradually made their way, until it is recognized that, on account of their delicacy, their directness, and their certainty, these methods now furnish the final criterion of the sanitary condition of a potable water.

A knowledge of the new science early became so indispensable for the sanitary expert that a special course in the Bacteriology of Water and Sewage has for some years been given to students of biology and sanitary engineering in the Biological Department of the Massachusetts Institute of Technology. For workers in this course the present volume has been especially prepared, and it is fitting, we think, that such a manual should proceed from an institution whose faculty, graduates, and students have had a large share in shaping the science and art of which it treats. We shall be gratified, however, if its field of usefulness extends to those following similar courses in other institutions, or occupied professionally in sanitary work.

The treatment of the subject in the many treatises on General Bacteriology and Medical Bacteriology is neither special enough nor full enough for modern needs. The classic work of Grace and Percy Frankland is now ten years old; and even Horrocks'

valuable "Bacteriological Examination of Water" requires to be supplemented by an account of the developments in quantitative analysis which have taken place on this side of the Atlantic.

It is for us a matter of pride that Water Bacteriology owes much of its value, both in exactness of method and in common-sense interpretation, to American sanitarians. The English have contributed researches of the greatest importance on the significance of certain intestinal bacteria; but with this exception the best work on the bacteriology of water has, in our opinion, been done in this country. Smith, Sedgwick, Fuller, Whipple, Jordan, and their pupils and associates (not to mention others) have been pioneers in the development of this new field in sanitary science. To gather the results of their work together in such form as to give a correct idea of the best American practice is the purpose of this little book; and this we have tried to do with such completeness as shall render the volume of value to the expert and at the same time with such freedom from undue technicality as to make it readable for the layman. It should be distinctly understood that students using it are supposed to have had beforehand a thorough course in general bacteriology, and to be equipped for advanced work in special lines.

Boston, *March* 10, 1904.

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ELEMENTS OF WATER BACTERIOLOGY

CHAPTER I

THE BACTERIA IN NATURAL WATERS

Bacteria and Their Nutritive Relations. Bacteria are the most numerous and the most widely distributed of living things. They are present not merely at the surface of the earth or in the bodies of water which partially cover it, as is the case with most other living things, but in the soil itself, and in the air above, and in the waters under the earth.

Like other organisms the bacteria are sensitive to external conditions, and respond quickly to slight changes in their environment. Temperature, moisture, and oxygen are of importance in controlling their distribution; but the most significant factor is the amount of food supply. Bacteria and decomposing organic matter are always associated, and for this reason a brief consideration of the general relation of bacteria to their sources of food supply must precede the study of their distribution in any special medium.

The bacteria possess greater constructive ability than any animal organisms. On the other hand, they lack the power of green plants to build up their own food from compounds like carbon dioxide and nitrates which have no stored potential energy. The food requirements of various bacterial types differ, however, widely among themselves. Fischer (1900) has divided the whole group into three great subdivisions according to the nature of their metabolism. The Prototrophic forms are characterized by minimal nutrient requirements, and include organisms like the nitrifying bacteria which require no complex organic compounds at all, but derive their nourishment from carbon dioxide or carbonates, nitrites and phosphates, or from inorganic ammonium

salts. A second group of Metatrophic bacteria includes those types which require organic matter, nitrogenous and carbonaceous, but are not dependent on the fluids of the living plant or animal. Finally, the Paratrophic bacteria are the true parasites, which exist only within the living tissues of other organisms. These sub-divisions, like all groups among the lower plants, are not sharply defined, and the metatrophic bacteria in particular exhibit every gradation, from types which grow in water with a trace of free ammonia to organisms, like the colon bacillus, which normally occur on the surface of the plant or animal body, feeding upon the fluids or on the extraneous material collected upon its surface and which may, under certain circumstances, invade the tissues themselves and produce disease.

The vast majority of bacteria belong to the second, or metatrophic group, living as saprophytes on dead organic matter wherever it may occur in nature, and particularly in that diffuse layer of decomposing plant and animal material which we call the humus, or surface layer of the soil. In this relation will be found the master key to the distribution of bacteria in water as well as in other natural habitats. It is true that certain peculiar forms may at times multiply in fairly pure waters; but, in general, large numbers of bacteria are found only in connection with the organic matter upon which they feed. Such organic matter is particularly abundant in the surface layer of the soil. Here, therefore, the bacteria are most numerous; and in air and water their numbers vary according to the extent of contact with the living earth.

Classification of Waters. Natural waters, then, group themselves from a bacteriological standpoint in four well-marked classes, according to their relation to the rich layers of bacterial growth upon the surface of the globe. There are first the *atmospheric waters* which have never been subject to contact with the earth; second, the *surface-waters* immediately exposed to such contamination in streams and pools; third, *stored waters* in lakes and large ponds where storage has reduced bacterial numbers and produced a state of comparative purity; and fourth, the *ground-waters* from which previous contamination has been even more completely removed by filtration through the deeper layers of the soil.

Bacterial Content of Various Waters. Even rain and snow, the sources of our potable waters, are by no means free from germs,

but contain them in numbers varying according to the amount of dust present in the air at the time of the precipitation. After a long-continued storm the atmosphere is washed nearly free of bacteria, so that a considerable series of sterile plates may often be obtained when 1-c.c. samples of such rain water are examined. These results are in harmony with the observations of Tissandier (reported by Duclaux, 1897), who found that the dust in the air amounted to 23 mg. per cubic meter in Paris and 4 mg. in the open country. After a rainfall these figures were reduced to 6 mg. and 0.25 mg., respectively.

With regard to what may be considered normal values for rain it is difficult to give satisfactory figures. Those obtained by Miquel (Miquel, 1886) during the period 1883–1886 showed on the average 4.3 bacteria per c.c. in the country (Montsouris) and 19 per c.c. in Paris. Snow shows rather higher numbers than rain. Janowski (Janowski, 1888) found in freshly fallen snow from 34 to 463 bacteria per c.c. of snow-water.

As soon as the rain-drop touches the surface of the earth its real bacterial contamination begins. Rivulets from ploughed land or roadways may often contain several hundred thousand bacteria to the cubic centimeter; and furthermore the amounts of organic and mineral matters which serve as food materials, and thus become a factor in later multiplication of organisms, are greatly increased.

In the larger streams several conditions combine to make these enormous bacterial numbers somewhat lower. Ground-water containing little microbic life enters as a diluting factor from below. The larger particles of organic matter are removed from the flowing water by sedimentation; many earth bacteria, for which an excess of water is an unfavorable medium, gradually perish; and in general a new condition of equilibrium tends to be established. It is difficult, however, to find a river in inhabited regions which does not contain several hundreds or thousands of bacteria to the cubic centimeter. Furthermore, heavy rains which introduce wash from the surrounding watershed may at any time upset whatever equilibrium exists, and surface-waters are apt to show sudden fluctuations in their bacterial content.

Seasonal Variation of Bacteria in Surface Waters. Sharp variations in bacterial content are particularly apt to occur in the spring and fall as a result of the rain and melting snow at those

seasons. The high numbers shown for various rivers in the table below illustrate this point.

SEASONAL VARIATIONS IN BACTERIAL CONTENT OF RIVER WATERS. BACTERIA PER C.C., MONTHLY AVERAGE

River	Year	Jan.	Feb.	Mar.	April	May	June
Thames [1]	1905–6	2,075	1,679	1,161	277	1,064	382
Lea [1]	1905–6	5,192	3.083	1,308	471	1,350	598
New [1]	1905–6	1,455	1,304	291	149	352	198
Mississippi [2]	1900–01	972	2,871	1,795	3,597	2,152	2,007
Potomac [3]	1906–7	4,400	1,000	11,500	3,700	750	2,300
Merrimac [4]	1905	14,200	14,800	10,300	3,600	1,900	9,600
Susquehanna [5]	1906	9,510	21,228	31,326	39,905	6,187	2,903

River	Year	July	Aug.	Sept.	Oct.	Nov.	Dec.
Thames [1]	1905–6	952	....	....	....	1,633	740
Lea [1]	1905–6	1,190	....	....	....	3,946	2,050
New [1]	1905–6	450	....	....	....	718	621
Mississippi [2]	1900–01	1,832	805	....	....	....	2,021
Potomac [3]	1906–7	2,700	3,000	6,200	2,300	1,800	6,900
Merrimac [4]	1905	3,900	19,500	13,500	39,800	8,700	.....
Susquehanna [5]	1906	685	1,637	836	7,575	26,224	37,525

[1] Houston, 1906*a*, 1906*b*.
[2] New Orleans, 1903.
[3] Figures obtained through courtesy of F. F. Longley.
[4] Massachusetts, 1906.
[5] Harrisburg, 1907.

The rainfall is the main factor which causes these seasonal variations; but its specific effect differs with different streams. The immediate result of a smart shower is always to increase contamination by introducing fresh wash from the surface of the ground. More prolonged moderate rain, however, exerts an opposite effect, and after the main impurities which can be washed away have been removed, may dilute the stream with water purer than itself. What the net effect of rain may be depends, therefore, on the character of the stream as well as on the character of the soil. A river of fairly good quality shows its highest numbers in rainy periods. With a highly polluted stream, on the other hand, the constant influx of sewage overbalances occasional contributions of surface contamination. Thus Gage (1906) shows in the following table that the bacterial content of the Merrimac is

highest when the stream is lowest, that is, when its sewage content is least subject to dilution.

VARIATIONS IN BACTERIAL CONTENT, MERRIMAC RIVER

GAGE (1906)

Flow of Stream. Cubic Feet per Second per Square Mile of Watershed	Bacteria per c.c.		Bact. coli per c.c.	
	Canal	Intake	Canal	Intake
Less than 1	7,500	10,800	66	88
1–2	6,800	6,200	50	51
2–4	3,600	5,600	29	39
Over 4	3,400	3,100	16	29

The contrast between the two classes of rivers is well brought out in a study of the Lahn and the Wieseck, by Kisskalt (1906); and the table below, compiled from his data, gives an excellent idea of the total numbers of bacteria and their seasonal fluctuations in a stream of fair quality (the Lahn) and a highly polluted one (the Wieseck). In the former case the bacterial numbers are highest when rain brings surface pollution; in the latter, when the sewage constantly present is least diluted.

MONTHLY VARIATIONS OF BACTERIA IN A NORMAL AND POLLUTED STREAM

KISSKALT, 1906

Date	Bacteria per c.c.		Date	Bacteria per c.c.	
1904	Lahn	Wieseck	1904–5	Lahn	Wieseck
July	318	104,000	December [1]	1,220	21,200
July	132	156,800	January [1]	3,668	29,920
August	840	98,400	February [1]	5,380	11,900
October [1]	1,235	28,400	March [1]	1,210	8,250
October [1]	420	58,000	April [1]	4,925	5,910
November	2,340	39,200	May	570	14,800
November [1]	1,740	52,000	June	686	50,180
December [1]	780	28,600			

[1] Rain or high water due to previous thaw.

Effect of Storage upon Bacteria in Water. In standing waters all the tendencies which make for the reduction of bacteria are intensified, and when a river passes into a natural or artificial reservoir a notable reduction in numbers occurs. The table below shows the striking effect produced upon the water of the Potomac River by its successive passage through the three reservoirs of the Washington water supply in the first nine months of 1907. We owe these figures to the courtesy of Mr. F. F. Longley, the engineer then in charge of the Washington filter plant.

REDUCTION OF BACTERIA IN WASHINGTON RESERVOIRS.
BACTERIA PER C.C., MONTHLY AVERAGE, 1907

	Potomac River	Dalecarlia Reservoir	Georgetown Reservoir	Washington City Reservoir
January	4,400	2,400	2,200	950
February	1,000	950	1,000	750
March	11,500	8,300	7,200	3,600
April	3,700	2,100	1,400	475
May	750	350	325	130
June	2,300	950	600	100
July	2,700	600	350	160
August	3,000	275	425	80
September	6,200	.....	1,900	230

The still more striking results obtained at London are indicated in the table below.

AVERAGE REDUCTION OF BACTERIA BY STORAGE AT LONDON
(Houston, 1909)

Water	Storage Days	Bacteria per c.c.		
		Gelatin 20°	Agar 37°	Bile-salt Agar 37°
Raw Thames River	.....	4465	280	41
Do. stored at Staines	95	175	34	2
Do. stored at Chelsea	15	208	44	5
Do. stored at Lambeth	14	362	52	8
Raw Lee River	.....	8135	382	34
Do. stored	58	67	11	1

When the water which enters a pond or a reservoir has already undergone considerable storage and reached a comparatively

stable condition, the diminution due to additional storage may be almost negligible. Thus Philbrick (1905) found that the influent water of the Chestnut Hill Reservoir of the Metropolitan Water Works of Boston contained on the average during the eleven years, 1893–1903, 220 bacteria per c.c., and the effluent 179. In many individual months, and in some whole years, the effluent contained more than the influent.

The seasonal variations in the bacterial content of a large pond or lake follow a somewhat different course from those observed in a stream. Philbrick, in the paper just cited, gives the figures tabulated below for the Chestnut Hill Reservoir of the Metropolitan Water Works (Boston). The averages are based on weekly analyses covering the eleven years, 1893–1903.

MONTHLY VARIATIONS IN BACTERIAL CONTENT OF CHESTNUT HILL RESERVOIR, 1893–1903

Month	J	F	M	A	M	J	J	A	S	O	N	D
Bacteria per c.c.	82	73	71	**123**	69	73	82	95	**134**	89	103	96

The marked increase in April and September is the notable feature of these analyses; and this is due to the effect of the spring and fall overturns which, in the months in question, stir up the decomposing organic matter at the bottom and distribute it through the reservoir. The slight, but steady, increase during the warm months from May to August is also probably significant.

On the whole it may be said that the bacterial content of a lake or pond should not be more than one or two hundred per c.c. and may often be under a hundred. The student will find numerous analyses of natural waters in Frankland's classic work (Frankland, 1894). He notes, for example, that the Lake of Lucerne contained 8 to 51 bacteria per c.c., Loch Katrine 74, and the Loch of Lintralthen an average of 170. The water of Lake Champlain examined by one of us (S. C. P.) in 1896 contained on an average 82 bacteria per c.c. at a point more than two miles out from the city of Burlington. Certain surface water-supplies near Boston studied by Nibecker and one of us (Winslow and Nibecker, 1903), gave the following results:

City	Number of Samples	Average Number of Bacteria per c.c.
Wakefield	7	59
Lynn	6	16
Plymouth	6	35
Cambridge	5	94
Salem	5	232
Medford	5	524
Taunton	4	13
Peabody	3	141

In sea-water, too, bacterial numbers are small, as noted by Russell at Naples (Russell, 1891) and Wood's Hole (Russell, 1892), and in salt as in fresh water the amount of bacterial life decreases in general as one passes downward from the surface and outward from the shore. Otto and Neumann (1904) obtained the results summarized below at various points on the high seas between Portugal and Brazil. Near the European coast numbers were much higher.

BACTERIA IN THE ATLANTIC OCEAN. (OTTO AND NEUMANN, 1904.) BACTERIA PER C.C.

Nearest Land	Depth in Meters			
	5	50	100	200
Canary Islands	120	76	20	1
Cape Verde Islands	58	16	64	6
St. Paul Island	20	480	54	4
Pernambuco	48	168	83	14

Drew (1912) finds high numbers of bacteria in surface sea-water off the Bahamas, ranging from 13,000 to 16,000, falling off below 200 fathoms (in the cold bottom waters at 10° C. or below) to 0 to 17.

Factors Influencing the Diminution of Bacteria in Surface-waters. The decrease in numbers which takes place when a surface-water is stored in a pond or reservoir indicates that the forces which tend to produce bacterial self-purification are important ones. It is necessary to consider in somewhat more detail just what these forces are, in order to gauge their potency in any particular instance.

Chief of them appear to be sedimentation, the activity of other micro-organisms, light, temperature, food-supply, and perhaps more obscure conditions such as osmotic pressure.

The subsidence of bacteria, either by virtue of their own specific gravity, or as the result of their attachment to particles of suspended matter, is unquestionably partly, if not largely, responsible for changes in the number of bacteria in the upper layers of water, at rest or in very sluggish streams. The results of numerous investigations by different workers seem to indicate that sedimentation of the bacteria themselves takes place slowly, and that the difference in numbers between the top layer and the bottom layer of water in tall jars in laboratory experiments of a few days' duration is very slight or quite within the limits of experimental error (Tiemann and Gärtner, 1889). Different species may, of course, be differently affected (Scheurlen, 1891). It must be remembered, however, that in natural streams bacteria are to a great extent attached to larger solid particles upon which the action of gravity is more important. Spitta (1903) found that from one-fifth to one-half of the bacteria in canal water may be attached to gross particles, as evidenced by their sedimentation in a few hours. Jordan (Jordan, 1900) is firmly of the opinion that in the lower part of the Illinois River, where there is a fall of but 30 feet in 225 miles, the influences summed up by the term sedimentation are sufficiently powerful to obviate the necessity for summoning another cause "to explain the diminution in numbers of bacteria," and he further adds: "It is noteworthy that all the instances recorded in the literature where a marked bacterial purification has been observed are precisely those where the conditions have been most favorable for sedimentation."

Little is known as to the share of other organisms in hastening the decrease of bacteria in stored water. Doubtless predatory Protozoa play some part in the process. Huntemüller (1905), after infecting water containing flagellate Protozoa with typhoid bacilli, found the Protozoa crowded with bacteria; and he observed under the microscope the actual ingestion of the living and motile bacilli. Korschun (1907) and others have obtained similar results and consider the activity of Protozoa to be an important factor in self-purification. Fehrs (1906) found that typhoid bacilli would live for 7 days in unsterilized Göttingen tap water, for 46 days in the same water sterilized, and for 13 days in water

inoculated with a culture of flagellate Protozoa after sterilization. Water bacteria were of course added with the Protozoa. Stokvis and Swellengrebel (1911) have shown that ciliated infusoria may also consume considerable quantities of bacteria under favorable conditions as to oxygen and temperature, and Hörhammer (1911) reports that certain Crustacea such as Cyclops may devour considerable quantities of typhoid bacilli when present in masses from cultures, stained with methylene blue, and suspended in water.

More recently the importance of Protozoa as a factor in reducing the bacterial content of polluted waters has been very clearly shown by Purdy and Butterfield (1918). These investigators found that bacterial numbers remained fairly constant in a sewage culture containing no protozoa, that protozoa (Paramœcia) died out rapidly in a bacteria-free sewage and that, where both bacteria and protozoa were present, the former increased rapidly at first and from two to six days later the bacterial numbers fell off as the protozoa increased.

Another factor of potential significance in the reduction of bacterial numbers in water is the bacteriophage of d'Herelle (1922). In fact the first clear record of bacteriophage action, according to d'Herelle is the observation of Hankin (1896) that the waters of certain Indian rivers exert a peculiar antiseptic action. Thus the water of the Jumna below Agra contains over 100,000 bacteria per c.c. while at a distance of 5 kilometers the numbers fall to less than 100. The antiseptic power of these waters is destroyed by boiling as indicated by the experiment with cholera vibrios tabulated below.

NUMBER OF VIBRIOS AFTER

	0 hours	1 hour	2 hours	3 hours	4 hours	25 hours	49 hours
Unheated water....	2500	1500	1000	500	0	0	0
Boiled water.....	5000	4000	6000	10,000	6000	10,000	36,000

Somewhat similar observations were made by Eliava in regard to the water of the Konra River at Tiflis and his results are particularly suggestive of bacteriophage action since the water vibrios in this case grew actively in peptone solution at first and were then killed out as shown by repeated examinations.

Certain bacteriologists have held that the toxic waste products of the bacteria themselves may render water unfit for their own development. Horrocks (Horrocks, 1901), Garré (Garré, 1887), Zagari (Zagari, 1887) and Freudenreich (Freudenreich, 1888) have shown that an "antagonism" exists when bacteria are grown in artificial culture media, such that the substratum which has supported the growth of one form may be rendered antiseptic to another. Frost (1904) has exhaustively studied the phenomenon of antagonism by exposing typhoid bacilli in collodion sacs to the action of certain soil and water bacteria growing in broth. Artificial culture media, however, offer conditions for bacterial development which are scarcely paralleled in natural waters. It is difficult to believe that under ordinary conditions poisons are produced of such power as to render a stream or lake specifically toxic for any particular type of bacteria. It does appear indeed from the experiments of Jordan, Russell and Zeit (1904) and Russell and Fuller (1906), which will shortly be referred to more fully, that the life of typhoid germs is shorter in water containing large numbers of other bacteria than in that of greater purity. Horrocks (1899), too, found freshly isolated typhoid bacilli alive in sterile sewage after 60 days, while they disappeared in 5 days when *Bact. coli* was also present. These phenomena may be due, however, to a struggle for oxygen, or for food, rather than to the assumed presence of highly toxic bacterial products, of which there is no independent evidence.

Many investigations conducted since the pioneer researches of Downes and Blunt (Downes and Blunt, 1877) have confirmed the results reported by them, which showed that direct sunlight is fatal to most bacteria in the vegetative state and even to spores if the exposure be sufficiently long, while diffused light is harmful in a less degree. Opinions vary as to the degree to which light is active in destroying the bacteria in natural waters. Buchner (Buchner, 1893) found by experiment that the bactericidal power of light extends to a depth of about three meters before it becomes imperceptible. On the other hand, Procaccini (Procaccini, 1893) found that when sunlight was passed vertically through 60 cm. of drain-water the lower layers contained nearly as many bacteria after 3 hours' treatment as before the exposure. The middle and upper portions showed a great falling off in numbers, however.

But few studies have been made of the effect of light on bacteria in flowing water. Jordan (Jordan, 1900) has investigated several Illinois streams and arrived at the conclusion that in moderately turbid water, at least, the sun's rays are virtually without action. On the other hand, Rapp has observed a considerable reduction of the bacteria in the Isar at Pullach after the period of diurnal insolation, as shown by the figures presented below. Clemesha (1912[a]) attributes very great importance to the action of light in the self-purification which takes place in Indian lakes and rivers; his opinion is apparently not based on comparative experiments including and excluding this factor, but chiefly on the greater numbers of intestinal bacteria at the bottom as compared with the superficial layers of water. Here, of course, sedimentation may also have had its effect.

EXAMINATIONS OF THE ISAR AT PULLACH

(RAPP, 1903)

(*A*) *Carried out September 26, 1898, no rain having fallen for three weeks*

Temperature		Time of the Experiment	Bacteria per c.c.
of the Water	of the Air		
13.0° C.	8.8° C.	7.30 P.M.	146
12.1° C.	7.0° C.	9.30 P.M.	270
10.5° C.	6.2° C.	5.00 A.M.	370
10.2° C.	8.2° C.	8.00 A.M.	320

(*B*) *Carried out November 28, 1898, no rain having fallen for some time*

5.5° C.	3.0° C.	6.00 P.M.	266
5.5° C.	2.5° C.	8.00 P.M.	402
5.5° C.	2.0° C.	10.00 P.M.	482
5.0° C.	2.0° C.	3.00 A.M.	532
4.5° C.	2.5° C.	7.30 A.M.	400

It is unnecessary to dwell in detail upon the effect which the lack of nutritive elements must exert upon intestinal bacteria and soil bacteria in waters of ordinary purity. Comparative studies of culture media, to be quoted in the succeeding chapter, will show how delicately the bacteria respond to comparatively slight changes in their food-supply. Wheeler (1906) found that

typhoid bacilli would persist in almost undiminished numbers in sterilized water from a polluted well containing considerable organic matter and kept in the dark at 20 degrees, while in purer water or in the light they died out in from 2 to 6 weeks.

Whipple and Mayer (1906) have called attention to another important factor in the general problem. They report that typhoid and colon bacilli survive in water better under an atmosphere of air than under an atmosphere of hydrogen; Hinds (1916), however, reports directly opposite results for *Bact. coli* with a somewhat different technique.

Various inorganic constituents of the medium undoubtedly exercise an important influence upon the life of bacteria in water; and the mutual interaction of the different substances present is a highly complex one. Thus Winslow and Lochridge (1906) report that five parts of dissociated hydrogen per million parts of tap water (0.005 normal HCl) is fatal to typhoid bacilli, while ten times as much acid is required for sterilization when 1 per cent of peptone is present to check the dissociation of the hydrogen. In Hazen and Whipple's study of the Allegheny, Monongahela and Ohio rivers at Pittsburgh the antiseptic effect of acid wastes was strikingly shown. (Engineering News, 1912). In connection with the important sanitary problem presented by the control of " soft drinks " it should be noted that carbonated beverages exert a distinct antiseptic action, which is markedly accentuated in those which also contain citric or lactic acids (Koser and Skinner, 1922).

The effect of various mineral salts upon bacterial viability has been exhaustively reviewed by Falk (1923). Jackson (1922) reports total counts of only a few hundred thousand bacteria per c.c. and *B. coli* counts of between one and two hundred per c.c. at points on the Naugatuck River where the pollution is so great that much larger numbers would be expected. Copper wastes from brass factories were chiefly responsible for this condition.

Although it is hard to estimate the exact importance of each factor, the general phenomena of the self-purification of streams are easy to comprehend. A small brook, immediately after the entrance of polluting material from the surface of the ground, contains many bacteria from a diversity of sources. Gradually those organisms adapted to life in the earth or in the bodies of plants and animals die out, and the forms for which water furnishes

ideal conditions survive and multiply. It is no single agent which brings this about, but that complex of little-understood conditions which we call the environment. If any one thing is of prime importance it is probably the food-supply, for only certain bacteria are able to multiply in the presence of the small amount of organic matter present in ordinary potable waters. As Jordan (Jordan, 1900) has said: "In the causes connected with the insufficiency or unsuitability of the food-supply is to be found, I believe, the main reason for the bacterial self-purification of streams."

Effect of Temperature upon Bacteria in Water. The effect of temperature upon the survival of bacteria in water varies according to this primary condition of food-supply which has just been discussed. When bacteria are in a medium in which they are able to grow and multiply, warmth, within reasonable limits of course, favors their development. At times this may be true even of certain intestinal bacteria in water. Thus at Harrisburg, Pa., a series of *Bact. coli* examinations made in the midsummer of 1906 showed positive results in 7 per cent of the samples of water entering the storage reservoir and in 27 per cent of the samples leaving it. The storage period in this case was about two days and the temperature of the water in the reservoir was nearly at blood heat (Harrisburg, 1907). Clemesha (1912[a]) has recently made an exhaustive study of this multiplication of coli-like microbes in warm waters and has shown that it is confined to certain particular types within the colon group. For most intestinal bacteria the conditions necessary for growth and multiplication are not realized in water and an entirely different temperature effect is manifest. When a bacterium cannot multiply, the only vital activity which can take place is a katabolic wasting away, which soon proves destructive, and the higher the temperature the more rapidly the fatal result is reached. A frog in winter lives at the bottom of a pond breathing only through its skin, and eating not at all, but as soon as the temperature rises it must eat and breathe through its lungs or perish. It is quite true that even in ice 40 per cent of typhoid bacilli perish in 3 hours, and 98 per cent in 2 weeks (Sedgwick and Winslow, 1902). Recent work has shown, however, that they die in spite of the cold, not on account of it, and that the decrease is more rapid at higher temperatures, unless of course food-supply and other conditions admit of mul-

tiplication. Houston (1911) has furnished a very clear demonstration of this temperature relation by storing typhoid bacilli in water.

EFFECT OF TEMPERATURE ON SURVIVAL OF TYPHOID BACTERIA IN WATER

(Houston, 1911)

Temperature C.	Percentage of Typhoid Bacilli Surviving after One Week	Period of Final Disappearance of Bacilli
0	46	9 weeks
5	14	7 weeks
10	0.07	5 weeks
18	0.04	4 weeks

Ruediger (1911) has shown that colon bacilli are far more abundant in the Red Lake River during the winter when the river is covered with ice than in summer, although the volume of the river and the amount of sewage pollution are about the same. Typhoid bacilli in celloidin dialyzers floated down the river showed only 2.5 and 3.5 per cent surviving in 2 days and 0.51, 0.89, 2.2 and 3.2 per cent surviving in 3 days when the river was not frozen, while dialyzers suspended through the ice in colder weather showed 6.1, 10.5, 17.7, 46.8 and 62.9 per cent surviving in five different experiments after 2 days, 31 per cent in 3 days, 19 per cent in 7 days, and 2.5 per cent in 14 days. Ruediger attributes this greater persistence at low temperatures to the absence of poisonous waste products of other organisms and to protection from the light; but there can be little doubt that it is mainly a result of the general preservative effect of cold. From an epidemiological standpoint the conclusion that disease germs perish quickly in warm waters is amply confirmed. Almost without exception outbreaks of typhoid fever due to polluted water occur in cold weather and this is, in part at least, due to the greater persistence of typhoid bacilli at low temperatures.

Relation between Time of Storage and Self-purification. It is obvious that the efficiency of all the agencies which tend to decrease the number of bacteria in surface waters will increase with the prolongation of the period for which they act. Time is the great measure of self-purification; and in a comprehensive study

of the self-purification of the Potomac River, the investigators of the U. S. Public Health Service have found that the time relation is close enough to be stated mathematically. Prof. Phelps expresses this in the formula $\log \frac{n_1}{n_2} = kt$, where t is the time and n_1 and n_2 the respective numbers of bacteria; k, of course, will vary with the temperature and the other conditions discussed above.

Cohen (1922) in an exhaustive study of the viability of colon and typhoid bacilli in water finds that the temperature coefficient for these two organisms is distinctly different, their relative resistance being as 67 is to 1 at 0° C. and as 8 to 1 at 30°. The viability of *Bact. typhosum* in water falls rapidly at pH values more acid than 5.0 or more alkaline than 6.4. For *Bact. coli* the zone of optimum pH is wider and centers about absolute neutrality. Cohen believes with Chick and Phelps that the rate of reduction in numbers follows the law of logarithmic decline and therefore corresponds to the curve of a monomolecular reaction. Falk in unpublished work carried on in the laboratories of the Yale Medical School has extensive data which indicate that this is by no means always the case and that the problem is in reality a highly complex one; yet for practical purposes the logarithmic rate of reduction is a sufficiently close approximation to the truth.

The absolute time required to make a polluted water safe will obviously vary with the value of k in the formula quoted above.

Jordan, Russell and Zeit (1904), in an important series of experiments, added typhoid bacilli to the unsterilized waters of Lake Michigan, the Chicago River and Drainage Canal and the Illinois River, in collodion sacs suspended in the respective bodies of water. From the relatively pure Lake Michigan water the specific organisms could be isolated for at least a week, but in the polluted waters of the rivers and the Drainage Canal they were not found after 3 days except in a single instance. Russell and Fuller (1906), confirmed these general results, finding that typhoid bacilli would live for 10 days in the unsterilized water of Lake Mendota, while they could be isolated only after 5 days when immersed in sewage. Other observers record much greater viability for the typhoid bacillus. Savage (1905) added a heavy dose of the organism to unsterilized tidal mud and found it living after 5 weeks. Hoffmann (1905), after inoculating a large aquarium

with a rich typhoid culture, was able to isolate the germ from the water after four weeks and from the mud at the bottom after two months. Konrádi (1904) reports the persistence of typhoid bacilli in unsterilized tap water for over a year.

These last experiments deal only with the maximum survival period for a few out of great numbers of germs introduced into the water or mud, and entirely ignore the quantitative aspects of the case. When one considers the proportion of the original bacteria surviving, the period necessary to bring about a reasonably safe condition is found to be much shorter. Houston (1908) has shown that when water is artificially infected with typhoid bacilli and stored, 99.9 per cent of the disease germs perish in one week, although some may persist for from 1 to 9 weeks.

In later experiments (Houston, 1911) he finds that "uncultivated" typhoid bacilli added to the water directly from the urinary sediment of a disease carrier perish much more rapidly than the laboratory strains, usually disappearing entirely after one week and always after three. On a number of occasions Dr. Houston gave dramatic expression to his confidence in these negative laboratory findings by drinking half pint portions of water which a few weeks previously had contained millions of typhoid bacilli. We have plenty of practical epidemiological evidence, such as that offered in the Chicago Drainage Canal case and in the lawsuit over the condition of the water supply of Jersey City, to confirm the general conclusion that any water which has been stored for 4 weeks is practically safe.

Bacteria in Ground-waters. In general we have seen that surface-waters tend continually to decrease in bacterial content after their first period of contact with the humus layer of the soil. In that other portion of the meteoric water which penetrates below the surface of the earth to join the reservoir of ground-water, later to reappear as the flow of springs and wells, this diminution is still more marked, since the filtering action of the earth removes not only most of the bacteria, but much of their food material as well. The numbers of bacteria in the soil itself decrease rapidly as one passes downward. Kabrhel (1906) found several million per c.c. in surface samples of woodland soil, a few thousands or tens of thousands half a meter below, and usually only hundreds in centimeter samples collected at depths greater than a meter. Many observers formerly believed that all ground-waters were

nearly free from bacteria, because often no colonies appeared on plates counted after the ordinary short periods of time. If, however, a longer period of incubation be adopted considerable numbers may be obtained.

For convenience we may divide ground-waters into three groups, namely: shallow open wells, springs and "tubular" (driven) or deep wells. This division is important because ordinary shallow wells form a group by themselves in respect to the possibility of aerial and surface contamination, their water often being fairly rich in bacterial life. Egger (Wolffhügel, 1886) examined 60 wells in Mainz and found that 17 of them contained over 200 bacteria to the cubic centimeter. Maschek (Maschek, 1887) found 36 wells out of 48 examined in Leitmeritz which had a bacterial content of over 500 per c.c. Fischer (Horrocks, 1901) reported 120 wells in Kiel which gave over 500 bacteria per c.c. and only 51 with less than that number.

In the examination of 147 shallow farmyard wells by one of us (S. C. P.) it was found that 124 of the wells which contained no *Bact. coli*, and were therefore probably free from fecal pollution, averaged 190 bacilli per c.c. while 23 which gave positive tests for *Bact. coli* averaged 570 per c.c. The distribution of the two series of samples according to the number of bacteria present is indicated in the table below.

BACTERIA IN SHALLOW FARMYARD WELLS

Percentage of Samples in Each Group

Bacteria per c.c.....	0	1–10	11–20	21–50	51–100	101–500	501–1000	1001–2000	2001–3000
Series I. Bact. coli absent.....	3	16	14	16	11	31	5	4	...
Series II. Bact. coli present.....	..	...	5	.....	10	57	10	14	5

Very similar results are reported for shallow wells used as farm water-supplies in Minnesota by Kellerman and Whittaker (1909), although the general quality of the wells examined was considerably below that of the series tabulated above.

In the ordinary standard 48-hour period very few bacteria develop from normal spring-waters. Thus in an examination

of spring-waters made by the Massachusetts State Board of Health in 1900 (Massachusetts State Board of Health, 1901), of 37 springs which were practically unpolluted and had less than 0.10 part per 100,000 excess of chlorine over the normal, 54 samples were examined and gave an average of 41 bacteria per c.c. Only 6 samples showed figures over 50.

It now remains to consider the other great division of groundwaters, namely, deep, "driven," or "tubular" wells, which, if carefully constructed, should ordinarily be free from all surface-water contamination, and should show low bacterial counts. The results tabulated below obtained by Houston in the examination of a series of deep wells of high quality at Tunbridge Wells are fairly typical.

BACTERIAL CONTENT OF DEEP WELL WATERS

(Houston, 1903)

Bacteria per c.c.

36	6	9	4	1
16	17	4	3	12
2	4	10	5	2

Fifteen driven wells in the neighborhood of Boston, examined in 1903, showed at the end of 48 hours an average of only 18 colonies per c.c.; and the results of certain examinations of other wells and springs, recently made by the authors, are given in the table below.

BACTERIA IN DEEP WELL AND SPRING WATERS

Town	Bacteria per c.c.	Town	Bacteria per c.c.
Worcester, Mass.....	10	Saranac Lake, N. Y....	11
Waltham, Mass.....	3	Ellenville, N. Y.....	0
Newport, R. I.....	7	Hyde Park, Mass.....	12

It is plain that water absolutely free from bacteria is not ordinarily obtained from any source. In deep wells, however, their number is small; and the peculiar character of the organisms present is manifested in many cases by the slow development at room temperature (frequently no growth until the third day),

the entire absence of liquefying colonies, and the abundance of chromogenic species.

The Types of Bacteria Found in Water. As a rule the majority of the bacteria isolated from water belong to a few fairly well marked groups (the Franklands, 1894; Ward, 1897; Fuller and Johnson, 1899; Jordan, 1903).

The more obvious of these groups are (*a*) the fluorescent bacteria; (*b*) the chromogenic bacteria, including violet, red and yellow forms; (*c*) organisms of the colon-aerogenes group; (*d*) organisms of the proteus group; (*e*) non-gas-forming, non-chromogenic, non-spore-forming rods which do not produce proteus colonies and may or may not acidify milk and liquefy gelatin; (*f*) spore-formers of the *Bact. subtilis* type; and (*g*) white, yellow and pink cocci. Group (*e*) appears to be largely represented in natural waters. Its biological homogeneity and relationship to groups (*c*) and (*d*) are by no means clear.

CHAPTER II

THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER

Relation of the Medium to the Number of Bacteria Obtained. The customary methods for determining the number of bacteria in water do not reveal the total bacterial content, but only a very small fraction of it, as becomes apparent when we consider the large number of organisms, nitrifying bacteria, strict anærobes, etc., which refuse to grow, or grow only very slowly in ordinary culture media, and which, therefore, escape detection. On the one hand, certain obligate parasites cannot thrive in the absence of the rich fluids of the animal body; on the other hand, the prototrophic bacteria, adapted to the task of wrenching energy from nitrites and ammonium compounds are unable to develop in the presence of so much organic matter. Winslow (1905) in the examination of sewage and sewage effluents, found 20–70 times as many bacteria by microscopic enumeration as by the gelatin plate count. Certain special media enable us to obtain much larger counts than those yielded by the ordinary gelatin method. The Nährstoff Heyden agar, for example, was once strongly advocated by Hesse (Hesse and Niedner, 1898) and other German bacteriologists upon such grounds. In this country Gage and Phelps (Gage and Phelps, 1902) showed that the numbers obtained by the ordinary procedure were only from 5 to 50 per cent of those obtained by the use of Heyden's Nährstoff agar. For practical sanitary purposes, however, our methods are fairly satisfactory. Within limits, it is of no great importance that one method allows the growth of more bacteria than another. When we are using the quantitative analysis as a measure of sewage pollution the essential thing is that the section of the total bacterial flora which we obtain should be thoroughly representative of that portion of it in which we are most interested — the group of the quickly growing, rich-food-loving sewage forms. In this respect meat-gelatin-peptone appears to be unrivalled; and it is in this respect

that such media as Nährstoff agar fail. Müller (1900) showed that the larger counts obtained by plating on the Nährstoff medium are due to the fact that it specially favors the more prototrophic forms, among the water bacteria themselves. Intestinal organisms and even the ordinary putrefactive germs, when plated in pure culture, show no higher counts on Nährstoff agar than on gelatin. Gage and Adams (1904) found by plating pure cultures of the common laboratory bacteria, saprophytes and parasites,

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON REGULAR AGAR AND NÄHRSTOFF AGAR FOR DIFFERENT CLASSES OF WATERS

(GAGE AND PHELPS, 1902)

Regular Agar

Class of Water	Days' Count						
	2	3	4	5	6	7	8
Ground water	0	5	6	6	6	6	6
Filtered water	6	7	7	7	7	7	7
Merrimac River	6	7	7	8	8	9	9
Filtered sewage	14	17	18	19	19	19	19
Sewage	34	44	46	46	46	46	46

Närhstoff Agar

Ground water	6	43	78	88	93	100	100
Filtered water	37	69	80	92	98	100	100
Merrimac River	29	78	93	97	97	99	100
Filtered sewage	26	65	93	95	97	99	100
Sewage	39	75	95	100	100	100	100

that Nährstoff counts were actually lower than those obtained by the use of gelatin. When sewage and highly polluted waters are examined counts are slightly higher on Nährstoff media, while with purer waters the Nährstoff numbers are far in excess of those obtained with gelatin. Winslow (1905) found the ratio of Nährstoff agar to gelatin count to be 1.7 to 1.0 for sewage, and 4.8 to 1.0 for sand filter effluent. With waters of still better quality the ratio goes even higher, reaching a maximum when the bacteria which increase and multiply in fairly pure water are most

abundant. Müller (1900) found, for example, that water which normally showed six times as many bacteria on Nährstoff agar as on gelatin might give a Nährstoff-gelatin ratio of 20–30 after it had been standing for some time in the supply pipes. The table on p. 22, taken from the valuable paper by Gage and Phelps (1902), shows strikingly the different Nährstoff-agar ratios for waters of various grades of purity. It is obvious from all these facts that the effect of using the Nährstoff medium is to increase disproportionately the bacterial counts obtained from purer waters and thus to diminish the difference in bacterial content between normal and contaminated sources. The ordinary agar and gelatin media, on the other hand, are adapted to the growth of intestinal and putrefactive forms and, therefore, serve best the prime object of bacteriological water examination.

The first requisite in a procedure for water analysis is, then, that it should be adapted to the end in view, the differentiation of pure and contaminated waters. The second and equally important requirement is that the procedure should be a standard one, so that results obtained at different times and by different observers may be comparable. In this respect the work of G. W. Fuller, G. C. Whipple, and other members of the Committee on Standard Methods of the American Public Health Association has placed the art of quantitative water analysis in this country in a very satisfactory state by contrast with the varying practices which have prevailed in England and Germany. The first report on this question was made in 1897 (Committee of Bacteriologists, 1898). A permanent Committee on Standard Methods was then formed which reported in 1901 (Fuller, 1902), in 1904 (Committee on Standard Methods of Water Analysis, 1905), in 1911 (Committee on Standard Methods for the Examination of Water and Sewage, 1912), in 1916 (Committee on Standard Methods for the Examination of Water and Sewage, 1917), in 1919 (Committee on Standard Methods for the Examination of Water and Sewage, 1920), and in 1922 (Committee on Standard Methods for the Examination of Water and Sewage, 1923), recommending in considerable detail a standard routine procedure for the quantitative and qualitative bacteriological examination of water for sanitary purposes. These reports have had a far-reaching effect in simplifying and unifying the methods of water analysis. Similar results have followed from the work

of the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water which reported in 1904, although this committee unfortunately did not consider the process of media making in great detail. The latest report of the American Committee on Standard Methods (1923) will be adhered to in this and succeeding chapters unless otherwise specifically stated.

Standard Procedure for Quantitative Determination of Bacteria in Water. The procedure for the quantitative determination of bacteria in water consists, in brief, in mixing a definite amount of a suitably collected specimen of the water with a sterile, solidifiable culture medium and incubating it for a sufficiently long time to permit reproduction of the bacteria and the formation of visible colonies which may be counted. The process is divided naturally into four stages — sampling, plating, incubating, and counting.

Sampling. All samples of water for bacteriological examination should be collected in clean, sterile bottles with wide mouths and glass stoppers, preferably of the flat mushroom type. It is desirable that these bottles should have a capacity of at least 100 c.c.

They should be cleaned thoroughly before using, by treatment with sulphuric acid and potassium bichromate or with alkaline permanganate of potash followed by sulphuric acid, rinsed in clean water, dried by draining, and sterilized by dry heat at 170° C. for at least an hour and a half, or by steam at 120° for 15 minutes. If not to be used immediately the neck and stopper should be protected against dust or other contamination by wrapping with tin foil or paper before sterilization. For transportation the bottle should be enclosed in a suitable case or box.

The greatest care must be taken that the fingers do not touch the inside of the neck of the bottle or the cone of the stopper, as the water thereby would become seriously contaminated and rendered unfit for examination. It is well known that bacteria are found abundantly upon the skin, and Winslow (Winslow, 1903) has shown that even *Bact. coli* is present upon the hands in a considerable number of cases.

In order to obtain a fair sample, great precautions must be taken, and these will vary with the different classes of waters to be examined and with local conditions. If a sample is to be taken

from a tap, the water should be allowed to flow at least five minutes (if from a tap in regular use) or for a longer period in case the water has been standing in the house-service system. In the small pipes, changes in bacterial content are liable to occur, certain species dying and others multiplying.

If a sample is to be taken from a pump similar precautions are necessary. The pump should be in continuous operation for 5 minutes at least, and preferably for half an hour before the sample is taken, in order to avoid excessively high numbers due to the growth of bacteria within the well and pump, the bacterial condition of the water as it passes through the ground being what we wish to determine. Thus Heræus (Heræus, 1886) in a well-water which had been but little used during the preceding 36 hours found 5000 organisms per c.c.; when the well was emptied by continuous pumping, a second sample, after an interval of half an hour, gave only 35. Maschek (Tiemann and Gärtner, 1889) obtained similar results, shown in the following table:

EFFECT OF PUMPING ON THE BACTERIAL CONTENT OF WELL-WATER

Well-water after continuous pumping for	fifteen minutes	458
" " " "	many hours	140
" later		68
" after continuous pumping for	fifteen minutes	578
" " " "	many hours	179
" later		73

After a proper interval of pumping the sample of a well-water may be collected from the pet-cock of the pump or from a near-by tap. With a hand-pump, such as is found in domestic shallow wells, the water is, of course, pumped directly into the sample bottle. The difficulties in securing an average sample from this latter source are often great, since if the flooring about the pump is not tight, as is often the case, continued pumping may wash in an unusual amount of surface pollution.

In sampling surface-waters, the greatest precautions must be observed to prevent contamination from the fingers. In still waters the fairest sample is one taken from several inches down, as the surface itself is likely to have dust particles floating upon it. The method most frequently recommended is to plunge the bottle mouth downward to a depth of a foot or so, then invert and allow the bottle to fill.

Whenever any current exists, the mouth of the bottle should be directed against it in order to carry away any bacteria from the fingers. If there is no current, a similar effect can be produced by turning the bottle under water and giving it a quick forward motion. In rapidly flowing streams it is only necessary to hold the bottle at the surface with the mouth pointed up-stream.

For taking samples of water at greater depths, a number of devices have been employed, all of which are fairly satisfactory. The essentials are, first, a weight to carry the bottle down to the desired depth, and, second, some method of removing the stopper when that depth is reached. The student will find one good form of apparatus described in Abbott's "Principles of Bacteriology" (Abbott, 1899); an admirable one was devised by Hill and Ellms (Hill and Ellms, 1898); and Thresh (1904) figures an ingenious device for the same purpose. Miquel and Cambier (Miquel and Cambier, 1902) and other authors recommend the use of a sealed glass bulb with a capillary tube which can be broken off at the desired moment.

Wilson (1920) describes a valuable form of deep water sampler operating on the same principle. Drew (1912) has devised an interesting sampling apparatus for use at great depths in the sea.

Ice may be sampled by breaking off suitable pieces and flaming them thoroughly before dropping them into a wide mouthed sterile bottle. Greenfield (1916) has described a special apparatus for the sampling of ice for bacteriological examination which is based on the principle of a coal sampler.

Changes in Bacterial Numbers after Sampling. As soon as a sample of water is collected its conditions of equilibrium are upset and a change in the bacterial content begins. Even in the purest spring-waters, which contain but few bacteria when collected, and in which the amount of organic matter is infinitesimal, enormous numbers may be found after storage under laboratory conditions for a few days or even a few hours. In some cases the rise in numbers is gradual, in others very rapid. The Franklands (Frankland, 1894) record the case of a deep-well water in which the bacteria increased from 7 to 495,000 in 3 days. Miquel (Miquel, 1891), from his researches, arrived at the conclusion that in surface-waters the rise is less rapid than in waters from deep wells or springs, and that in the latter case the decrease, after reaching a maximum, is likewise rapid and steady. Just how far protection

from light, increase in temperature, and a destruction of higher micro-organisms is responsible for the increase, and to what extent an exhaustion of food-supply or the formation of toxic waste products causes the succeeding decrease, we are not aware; but the facts are well established.

Whipple has exhaustively studied the details of this multiplication of bacteria in stored waters and has shown in the table given below that there is first a slight reduction in the number present, lasting perhaps for 6 hours, followed by the great increase noted by earlier observers. It is probable that there is a constant increase of the typical water bacilli, overbalanced at first by a reduction in other forms, for which the environment is unsuitable.

BACTERIAL CHANGES IN WATER DURING STORAGE

(WHIPPLE, 1901)

Sample	Initial Temperature	Temp. of Incubation of Sample	Number of Bacteria per c.c.				
			Initial	After 3 Hours	After 6 Hours	After 24 Hours	After 48 Hours
	C.	C.					
A	7.6°	17.0°	260	215	230	900	27,000
B	7.6°	17.0°	260	245	255	720	10,850
C	7.6°	12.5°	260	270	231	600	2,790
D	7.6°	12.5°	260	270	245	710	1,800
E	7.6°	2.4°	260	243	210	675	1,980
F	7.6°	2.4°	260	235	270	560	1,980
G	11.0°	12.8°	77	55	58	101	10,250
H	11.0°	12.8°	77	53	74	87	2,175
I	11.0°	23.6°	77	51	52	11,000	41,400
J	6.7°	20.0°	430	375	245	.....	385,000[1]
K	6.7°	20.0°	430	345	405	.....	750,000[1]
L	23.2°	23.0°	510	340	230	8,000	20,000
M	23.2°	2.5°	525	300	220	380	2,200

[1] 0.0005 per cent peptone added to the water.

Wolffhügel and Riedel (Wolffhügel and Riedel, 1886) noted the dependence of this multiplication on the air-supply, vessels closed with rubber stoppers showing lower numbers than those plugged with cotton. Similarly, Whipple found that the multiplication of bacteria was much greater when bottles were only half full than when they were filled completely; and also, as shown in the

very striking table here given, that the size of the bottle markedly influenced the growth.

EFFECT OF SIZE OF VESSEL UPON THE MULTIPLICATION OF WATER BACTERIA DURING STORAGE

(WHIPPLE, 1901)

Sample	Bottle	Temp. of Incubation	Number of Bacteria per c.c.					
			Initial[1]	After 3 Hrs.	After 6 Hrs.	After 12 Hrs.	After 24 Hrs.	After 48 Hrs.
		C						
A	1-gallon	13°	77	63	65	47	42	175
B	2-quart	13°	77	59	63	60	45	390
C	1-quart	13°	77	63	63	47	46	325
D	1-pint	13°	77	57	61	36	38	630
E	2-ounce	13°	77	55	58	47	101	10,250
F	1-gallon	24°	77	81	97	275	290	300
G	2-quart	24°	77	92	59	62	180	250
H	1-quart	24°	77	84	77	46	340	900
I	1-pint	24°	77	51	46	100	2,950	7,020
J	2-ounce	24°	77	51	52	145	11,000	41,400

[1] Average of five plates.

An important series of investigations by Kohn (1906) suggests that this phenomenon of multiplication during storage may be due in part to the solution of certain constituents of glass which favor bacterial life, since the increase is notably greater in bottles of the more soluble glasses.

Whipple's table, quoted above, shows that the multiplication during storage was greater at a higher temperature; and this is a well-recognized general rule. In order to obviate the abnormal results of storage increase it is therefore obvious that samples must be examined shortly after collection and that they must be kept cool during their necessary storage. If fairly pure waters are placed upon ice and kept between 0 degrees and 10 degrees, they will show no material increase in 12 hours. With polluted water, however, another danger is here introduced. Samples of such water when packed in ice show a marked decrease due to the large number of sensitive intestinal bacteria present. Jordan (Jordan, 1900) found that three samples of river-water packed in ice for 48 hours fell off from 535,000 to 54,500; from 412,000 to

50,005, and from 329,000 to 73,000, respectively. It is, therefore, important that even iced samples should not be kept too long; and it is desirable to adhere strictly to the recommendations of the Standard Methods Committee that the interval between sampling and examination should not exceed 12 hours in the case of relatively pure waters, 6 hours in the case of relatively impure waters, and 1 hour in the case of sewage.

Plating. The bottle containing the sample of water is first shaken at least twenty-five times in order to get an equal distribution of the bacteria. If the number of bacteria present is probably not greater than 200, 1 c.c. is then withdrawn with a sterile 1 c.c. pipette and delivered into a sterile Petri dish of 10 cm. diameter. To this is added 10 c.c. of standard 10 per cent gelatin at a temperature of about 30° C., or standard agar at 40–42° C. Should the number of bacteria per c.c. probably exceed 300, dilution is necessary. This is best accomplished by adding 1 c.c. of the water in question to 9, 99 or 999, etc., c.c. of sterile tap water according to the amount of dilution required. The diluted sample is then shaken thoroughly and 1 c.c. taken for enumeration. In order to determine the number of bacteria originally present it is only necessary to multiply by the factor 10, 100, or 1000, etc.

When a sample of water from an unknown source is to be examined it is generally desirable to make two or more check plates at each of the above dilutions, selecting those which give between 30 and 300 colonies on the plates after incubation as the ones on which to rely for the count. A smaller number will not give average figures, and if more than 300 colonies are present on a plate many bacteria will be checked by the waste products of those which first develop and the count obtained will be too low. After the addition of the diluted sample and the nutrient medium, their thorough mixture in an even layer on the bottom of the plate is obtained by careful tipping and rotation.

It was formerly customary to mix the water with the gelatin in the tube before pouring into the plate, but this method is objectionable because there is always a residuum of medium remaining in the tube which will retain varying numbers of bacteria and thus interfere with the accuracy of the count. Before pouring the medium into the plate the mouth of the tube should be flamed to remove any possibility of contamination.

The usual method of determining the number of bacteria in water for sanitary purposes in Germany, England and the United States has always been by the use of gelatin plates with a 2-day incubation period at 20 degrees. The 1905 Standard Methods Report of the American Public Health Association Committee recommended this procedure, which was universally adopted. The 1912 Report, however, suggested the use of agar with a 1-day period at 37 degrees, as yielding quicker results and indicating the presence of bacteria more nearly related to pathogenic types. The comparative value of the two methods has been well discussed by Whipple (1913). The 1923 Standard Methods Reports wisely include both the 20° and 37° counts. Each determination yields results which are of significance in estimating the sanitary quality of a water supply; and neither can wholly supersede the other. It is the 20° count which will be discussed in this chapter, leaving the body temperature count for consideration in Chapter IV.

Composition of Medium. The exact composition of the medium is, of course, of prime importance in controlling the number of bacteria which will develop. The figures previously cited in connection with the discussion of Hesse's Nährstoff agar show how bacterial counts may vary with media of widely different composition. The table quoted on page 31 from Gage and Phelps (1902) shows the considerable differences which may be due to the presence or absence of meat infusion, peptone, etc., in media of generally similar character (compare the figures for plain gelatin, peptone, gelatin, and meat gelatin). Much slighter variations than this, however, are significant. The reaction of the medium was found as early as 1891 to be important, for Reinsch (Reinsch, 1891) showed in that year that the addition of one one-hundredth of a gram of sodium carbonate to the liter increased sixfold the number of bacteria developing. Fuller (Fuller, 1895) and Sedgwick and one of us (Sedgwick and Prescott, 1895), working independently, established the fact that an optimum reaction existed for most water bacteria and that a deviation either way decreased the number of colonies developing.

Bunker and Schuber (1922) criticise, forcibly and justly, the indefinite standards employed in defining the reaction of culture media in the past and even carried on into the 1920 edition of standard methods. They show that the reaction of the same

medium varies widely in different laboratories and urge that all media should be adjusted by the colorimetric method to a definite hydrogen ion concentration. They recommend pH 6.8–7.2 for ordinary solid and liquid media and pH 8.0–8.2 for Endo agar, and believe that for small laboratories the Hynson, Westcott and Dunning color standards may be used while the procedure of Medalia (1920) will be found more satisfactory for very accurate work. We are ourselves heartily in favor of these recommendations and believe that the old phenolphthalein method of titration for adjusting the reaction of culture media should be definitely abandoned in favor of adjustment to a pH near 7.0 by the use of phenol red or bromthymol blue. This step has at last been formally endorsed by the Committee on Standard Methods in its 1923 Report.

Gillespie (1921) has recommended a procedure essentially similar to that of Medalia and supported by more fundamental theoretical reasoning. The serious student of this problem of hydrogen ion concentration should familiarize himself with the classic text of Clark (1922).

TABLE SHOWING PERCENTAGES OF BACTERIA DEVELOPING ON MEDIA OF DIFFERENT COMPOSITIONS

(Gage and Phelps, 1902)

Medium	Days' Count							
	2	3	4	5	6	7	8	9
Nährstoff agar	19	60	78	85	95	99	99	100
Nährstoff peptone agar	10	22	26	28	30	30	30	30
Peptone agar	11	16	22	23	24	24	24	24
Meat agar	8	13	16	17	17	17	17	17
Plain agar	8	10	13	14	14	14	14	14
Regular agar	7	9	11	11	11	11	11	11
Nährstoff glycerin agar	6	10	11	11	11	11	11	11
Nährstoff meat agar	7	7	8	8	10	10	10	10
Meat gelatin	12	19	24	26	26	26	26	26
Peptone gelatine	7	12	18	20	20	20	20	20
Standard gelatin	8	10	11	12	13	13	13	13
Plain gelatin	1	6	12	13	13	13	13	13
Nährstoff gelatin	5	6	9	11	13	13	13	13

Whipple (Whipple, 1902) has shown that not only the particular kind of gelatin used, but its exact physical condition as affected by sterilization and other previous treatments, will materially affect the results obtained. Gage and Adams (1904)

found marked differences in counts as the result of the use of the two best-known commercial peptones. A long series of waters plated on agar made up with Merck's and Witte's peptones, respectively, showed the average relative results in the table below.

AVERAGE RELATIVE NUMBER OF BACTERIA ON PEPTONE AGAR WITH DIFFERENT PEPTONES

(Gage and Adams, 1904)

Days	2	4	6	8	10	12
Merck's.....	9	33	51	67	89	98
Witte's.....	38	53	100	100	100	100

The same authors showed that the composition of the water used exercised a marked selective action upon the development of bacteria. Agar made up with sewage permitted a maximum growth of sewage bacteria and showed no colonies when inoculated with filtered city water. On the other hand agar made up with city water showed 100 per cent of the bacteria present in city water and river water, three-quarters of those present in sewage and less than half of those present in sewage effluents.

All these facts make it evident that only the strictest adherence to a standard method can ensure comparable results; the ordinary nutrient gelatin or agar should then in all practical sanitary work be made up from distilled water, beef extract, peptone and gelatin or agar, in exact accordance with the directions of the Standard Methods Committee. The Committee took a distinct step forward in recommending Liebig's meat extract or any other brand shown by comparative tests to give equivalent results in place of the time consuming and inconstant meat infusion. For a peptone the Armour, Digestive Ferments Company, or Fairchild product or any other which gives equivalent results may be used. The gelatin must be of such spissitude that a 10 per cent medium shall have a melting point of 25° C. or over; and it must be dried for half an hour at 105° C. before it is weighed out. The use of Difco and other dehydrated culture media is permitted by the new Standard Methods, "provided these media give results equivalent to freshly prepared media."

Incubation. Incubation should take place in a dark, well-ventilated chamber where the temperature is kept substantially

constant at 20 degrees and where the atmosphere is practically saturated with moisture. It has been shown by Whipple (Whipple, 1899) and others that the number of bacteria developing in plate cultures is to a certain extent dependent upon the presence of abundant oxygen and moisture. Thus, reckoning the number of bacteria developing in a moist chamber at 100, the percentage counts obtained in an ordinary incubator were as follows: 75 when the relative humidity of the incubator was 60 per cent of saturation; 82 when it was 75 per cent; 98 when it was 95 per cent. This source of error may be avoided by the use of ventilated dishes and by the presence of a pan of water in the incubating chamber.

EFFECT OF THE LENGTH OF INCUBATION OF WATER BACTERIA IN GELATIN UPON THE NUMBER OF COLONIES DEVELOPING

(Miquel and Cambier, 1902)

Length of Incubation	Colonies Developed	Length of Incubation	Colonies Developed
1 day	20	9 days	821
2 days	136	10 days	859
3 days	254	11 days	892
4 days	387	12 days	921
5 days	530	13 days	951
6 days	637	14 days	976
7 days	725	15 days	1000
8 days	780		

According to American and German practice, plates made for sanitary water analysis are counted at the end of 48 hours. The English Committee appointed to consider the standardization of methods for the Bacterioscopic Examination of Water (1904) fixed the time at 72 hours. French bacteriologists, and some Germans (Hesse and Niedner, 1906), recommend still longer periods, and the preceding table from Miquel and Cambier (Miquel and Cambier, 1902) shows that many bacteria fail to appear in our ordinary procedure. It is, however, in the main, the characteristic water bacteria which develop slowly, sewage bacteria almost without exception being rapid growers. The longer period of incubation is, therefore, not only inconvenient, but undesirable, since it obscures the difference between good and bad waters.

A very interesting point bearing particularly on sewage bacteriology is brought out by Sturges (1919), who shows that the bacteria which survive treatment of sewage with copper salts or sulphur dioxide are so affected that their development on plates is greatly delayed. Counts made after the usual periods will therefore yield unduly low results. Sewage treated with chlorine apparently does not exhibit this phenomenon, at least, not to the same extent.

Counting. The number of bacteria is determined by counting the colonies developed upon the plate, with the aid of a lens magnifying $2\frac{1}{2}$ diameters, focal distance, $3\frac{1}{2}$ inches. For convenience in counting the plate may be placed upon a glass plate ruled in centimeter squares and set over a black tile; or the tile itself may be ruled. As has already been said, it is desirable that the number of colonies should not be too great, for when the number is very high the colonies grow only to a small size, making counting laborious and inaccurate, and many do not develop at all.

It is generally assumed that the best results are obtained when the colonies on a plate are between 30 and 300; and the Committee on Standard Methods recommends that where two plates are available with colony numbers within this range plates at other dilutions should be discarded. It may be noted, however, that Stein (1918) concludes that 200–400 colonies per plate yield the highest results.

Ayers (1911) has suggested two counting devices which will be found very useful where a great many plates have to be handled. For getting the best possible transmitted light, he places his plate on the ground-glass top of a wooden box, 7 inches square, with one side open to admit light, which is reflected upward by a plane mirror set in the box at an angle of 45 degrees. An ordinary graduated-glass counting plate may be placed between the ground-glass and the Petri dish, and the eyes are protected from direct light by a screen rising from the open side of the box. For picking colonies from a gelatin plate in a warm room, he places between the ground glass and the Petri dish a copper box with top and bottom of glass, 7 inches square and $1\frac{1}{2}$ inches deep, through which cold water is allowed to circulate.

Expression of Quantitative Results. It is customary in determining numbers to make plates in duplicate, thereby affording a check upon one's own work.

Stein (1918), who has analyzed the results of the bacterial plate count with regard to the probable errors involved, concludes that from three to ten platings should be made from a single sample to give a high degree of accuracy. It may perhaps be questioned whether such great accuracy in the enumeration of bacteria in each individual sample is worth while in view of the wide chance variation which necessarily exists between the actual bacterial content of different samples.

It should be possible for careful manipulators to obtain results within 10 per cent of each other, but a closer agreement than this is hardly to be expected. It has been suggested by the committee of the American Public Health Association that the following mode of expressing results be adopted in order to avoid the appearance of a degree of accuracy which the methods do not warrant.

NUMBERS OF BACTERIA FROM				
1–50	shall be recorded to the nearest unit			
51–100	"	"	"	5
101–250	"	"	"	10
251–500	"	"	"	25
501–1000	"	"	"	50
1001–10,000	"	"	"	100
10,001–50,000	"	"	"	500
50,001–100,000	"	"	"	1,000
100,001–500,000	"	"	"	10,000
500,001–1,000,000	"	"	"	50,000
1,000,001–5,000,000	"	"	"	100,000

The determination of numbers of bacteria in water in the field has frequently been attempted. Since the laboratory method of " plating out " is difficult to use in field work, the Esmarch tube process has often been employed. This consists in introducing into a tube of melted gelatin or agar 1 c.c. of the water and then rotating the tube until the medium has solidified in a thin layer on the inner wall. Other bacteriologists have devised ingenious field kits for adapting the plate method to this purpose, of which one very good form has been described by Van Buskirk (1912). The opportunity for air infection in work done outside a proper laboratory is, however, always great; and it is almost impossible to secure proper conditions for incubation in any makeshift establishment. On the whole, the authors are of the opinion that laboratory examinations are to be preferred to those made in the

field, if a laboratory can be reached within 12 hours or so of the time of collection of the samples.

EFFECT OF STORAGE ON BACTERIAL CONTENT

Place Collected	Total Count Agar, 37 Deg. C., 24 hrs.	Total Count Gelatine 20 Deg. C., 48 hrs.	Bact. coli per c.c.		
			Lactose Bile	Lactose Broth	Dextrose Bile
Time — immediately					
River	600	3,000	0.4	6.0	6.0
Settling basin	36	250	0	0.2	0.2
Tap at 1323 Ky	11	1,500	0	0	0
Blackmar Well	18	5	0	0	0
Well at 1338 Ohio	7	35	0.2	0	0
Time — 12 hours					
River	820	28,000	0.1	2.0	2.0
Settling basin	50	2,000	0.1	0.2	0.2
Tap at 1323 Ky	25	500	0	0	0
Blackmar Well	30	250	0	0	0
Well at 1338 Ohio	10	80	0.1	0.1	0
Time — 48 hours					
River	600	5,000	0.1	0.4	0.4
Settling basin	20	1,800	0	0.2	0.2
Tap at 1323 Ky	40	10,000	0.1	0.1	0
Blackmar Well	12	350	0	0	0
Well at 1338 Ohio	10	50	0.1	0.2	0.2

The Kansas State Water Survey has made use of a device for shipping iced samples which has given excellent results. The outfit is described in the American Journal of Public Health for May, 1914, and consists essentially of an insulated container in which the bottles are firmly held by a simple mechanism. With proper icing the samples are maintained at a temperature below 10 degrees C. for two or three days or even more. Very little change due to storage is found in the counts on agar when plates are incubated at 37 degrees C. and in winter the 20-degree counts are satisfactory on agar. Gelatine counts were found to be erratic and not dependable.

The preceding tables kindly sent us by Prof. C. C. Young show the comparative numbers obtained in the laboratory after certain periods of storage, and in the laboratory as compared with the actual field analyses.

CHAPTER III

THE INTERPRETATION OF THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION

Standards for Potable Water. The information furnished by quantitative bacteriology as to the antecedents of a water is in the nature of circumstantial evidence and requires judicial interpretation. No absolute standards of purity can be established which shall rigidly separate the good from the bad. In this respect the terms " test " and " analysis " so universally used are in a sense inappropriate. Some scientific problems are so simple that they can be definitely settled by a test. The tensile strength of a given steel bar, for example, is a property which can be determined. In sanitary water examination, however, the factors involved are so complex, and the evidence necessarily so indirect, that the process of reasoning much more resembles a doctor's diagnosis than an engineering test.

The older experimenters attempted to establish arbitrary standards, by which the sanitary quality of a water could be fixed automatically by the number of germs alone. Thus Miquel (Miquel, 1891) published a table according to which water with less than 10 bacteria per c.c. was " excessively pure," with 10 to 100 bacteria, " very pure," with 100 to 1000 bacteria, " pure," with 1000 to 10,000 bacteria, " mediocre," with 10,000 to 100,000 bacteria, " impure," and with over 100,000 bacteria, " very impure." Few sanitarians would care to dispute the appropriateness of the titles applied to waters of the last two classes; but many bacteriologists have placed the standard of " purity " much higher. The limits set by various German observers range, for example, from 50 to 300. Dr. Sternberg (Sternberg, 1892), in a more conservative fashion, has stated that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion, and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage. This is

probably as satisfactory an arbitrary standard as could be devised, but any such standard must be applied with great caution. The source of the sample is of vital importance in the interpretation of analyses; a bacterial count which would condemn a spring might be quite normal for a river; only figures in excess of those common to unpolluted waters of the same character give an indication of danger. Furthermore, the bacteriological tests are far more delicate than any others at our command, very minute additions of food material causing an immense multiplication of the microscopic flora. This delicacy necessarily requires, both in the process of analysis and the interpretation of results, a high degree of caution. As pointed out in the previous chapter, the touch of a finger or the entrance of a particle of dust may wholly destroy the accuracy of an examination. Even the slight disturbance of conditions incident upon the storage of a sample after it has been taken may in a few hours wholly alter the relations of the contained microbic life. It is necessary, then, in the first place, to exercise the greatest care in allowing for possible error in the collection and handling of bacteriological samples; and in the second place, only well-marked differences in numbers should be considered significant.

In the early days of the science, discussion ran high as to the interpretation of bacteriological analysis; and particularly as to the relation of bacterial numbers to the organic matter present in a water. Different observers obtained inconsistent results, and Bolton (Bolton, 1886) concluded that there was no relation whatever between the organic pollution of a water and its bacterial content. Tiemann and Gärtner (Tiemann and Gärtner, 1889) furnished the key to the difficulty in their statement that there are two classes of bacteria, the great majority of species normally occurring in the earth or in decomposing organic matter, which require abundance of nutriment, and certain peculiar water bacteria which can multiply in the presence of such minute traces of ammonia as are present in ordinary distilled water. Even these prototrophic or semi-prototrophic forms, however, require a definite amount of food of their own kind.

Kohn (1906) determined the minimal nutrient material requisite for certain of them and found that they could develop in the presence of 198×10^{-10} to 198×10^{-13} per cent of dextrose, 66×10^{-13} to 66×10^{-17} per cent ammonium sulphate and

66×10^{-13} to 66×10^{-19} per cent ammonium phosphate. Similar minute amounts of organic matter are found in the purest of natural waters and under exceptional conditions certain species of bacteria may therefore multiply in bottled samples, or, at times, in a well or the basin of a spring. In normal surface-waters, such growths of the prototrophic forms do not apparently occur. Here it is found as a matter of practical experience that the number of bacteria present depends upon the extent to which the water has been contaminated with decomposing organic matter, either by pollution with sewage or by contact with the surface of the ground. The bacterial content varies as the extent and character of the contamination varies. It measures not merely organic matter, but organic matter in a state of active decay, and like the ammonias and other features of the sanitary chemical analysis, indicates fresh organic pollution, with the added advantage that the presence of the stable nitrogenous compounds often present in peaty waters introduces no error in the bacteriological analysis.

Bacterial Content of Surface-waters. In judging of a surface-water the student will be aided by reference to the figures given for certain normal sources in Chapter I; the Boston tap water with 50 to 200 bacteria per c.c. (Philbrick, 1905), and the water of Lake Zurich with an average of 71 in summer and 184 in winter (Cramer, 1885), may be taken as typical of good potable waters; and numbers much higher than these are open to suspicion, since all contamination whether contributed by sewage or by washings from the surface of the ground is a possible source of danger. The excess of bacteria in surface-waters during the spring and winter months is by no means an exception to the general rule that high numbers are significant, since the peril from supplies of this character is clearly shown by the spring epidemics of typhoid fever which at the times of melting snow visit communities making use of unprotected surface-waters. Streams receiving direct contributions of sewage exhibit a similar excess of bacteria at all times, numbers rising to an extraordinary height near the point of pollution and falling off below, as the stream suffers dilution and the sewage organisms perish. Miquel (Miquel, 1886) records 300 bacteria per c.c. in the water of the Seine at Choisy, above Paris; 1200 at Bercy in the vicinity of the city, and 200,000 at St. Denis after the entrance of the drainage of Paris. Prausnitz (Prausnitz, 1890) found 531 bacteria per c.c. in the Isar above

Munich, 227,369 near the entrance of the principal sewer, 9111 at a place 13 kilometers below the city, and 2378 at Freising, 20 kilometers further down. Jordan (Jordan, 1900), in his study of the fate of the sewage of Chicago, found 1,245,000 bacteria per c.c. in the drainage canal at Bridgeport, 650,000 at Lockport, 29 miles below, and numbers steadily decreasing to 3660 at Averyville, 159 miles below the point of original pollution. Below Averyville the sewage of Peoria enters and the numbers rise to 758,000 at Wesley City, decreasing to 4800 in 123 miles of flow to Kampsville. Brezina (1906) found 1900 bacteria per c.c. in the Danube River above, and 110,000 at the mouth, of the Danube canal. This number fell to 85,000 one kilometer below, 62,000 four kilometers below, and 40,000 seven kilometers down the stream. Vincent (1905) records from 1000 to 46,000 bacteria per c.c. in the waters of more or less polluted French rivers. Mayer (1902), on the other side of the world, found 21 and 35 bacteria per c.c. in the Shaho River, near its source, in the vicinity of the great Chinese Wall and from 100,000 to 600,000 in the highly polluted Whangpo near its mouth.

Bacterial Content of Ground-waters. In ground-waters we have seen that bacteria may occasionally be present in considerable numbers, but if so they are generally organisms of a peculiar character, incapable of development on the ordinary nutrient media in the standard time. Thus in 48 hours we often obtain counts measured only in units or tens such as have been recorded in Chapter I. When higher numbers are present, the general character of the colonies must be taken into account, since besides the slowly-growing forms certain other water bacteria, which require a comparatively small amount of nutriment, may multiply at times in a deep well or the basin of a spring. In such a case, however, the appearance of the plates at once reveals the peculiar conditions, for the colonies are of one kind and that distinct from any of the sewage species. Thus Dunham (Dunham, 1889) reports that the mixed water from a series of driven wells gave 2 bacteria per c.c., while another well, situated just like the others, contained 5000, all belonging to a single species common in the air. Except in such peculiar cases as this, high numbers in a ground-water mean contamination.

Bacteria in Filtered Waters. The process of slow sand filtration for the purification of unprotected surface-water is essentially

similar to the action which takes place in nature when rain soaks through the ground to appear in wells and springs; and it is in the examination of the effluent from such municipal plants that the quantitative bacteriological analysis finds, perhaps, its most important application. The chemical changes which occur in the passage of water through sand at a rate of 1,000,000 or 2,000,000 gallons per acre per day are so slight as to be negligible. The bacteria present should, however, suffer a reduction of 98 or 99 per cent, and their numbers furnish the best standard for measuring the efficiency of such filtration plants. At Lawrence, in 1905, Clark found an average of 12,700 bacteria per c.c. in the raw water of the Merrimac River, while the number present in the filtered water was only 70 (Massachusetts State Board of Health, 1906). Where the number of bacteria in the applied water is smaller it is difficult to obtain so high a percentage efficiency. At Washington, for example, prolonged sedimentation generally reduces the bacterial numbers to less than a thousand and it is almost impossible to secure a 99 per cent removal. The actual numbers of bacteria in the effluent are, however, much lower than at Lawrence. The monthly average results obtained for a year at these two plants are tabulated on page 42.

It must of course be remembered that the bacteria in the effluent of a filter do not all come through the filter with the water which is being treated. A substantial proportion, and where purification is high a large proportion, of these bacteria have been contributed by growths of metatrophic forms in the lower layers of the filter and in the underdrains. The contribution from the underdrains will be lower in winter than in summer, and with increasing rates of filtration the number of bacteria coming through with the water will steadily increase while the number (per c.c. of effluent) contributed by the underdrains will steadily decrease (Hazen, 1900).

Mechanical filtration of water gives results very like those obtained by slow sand treatment. Fuller at Cincinnati (Fuller, 1899) records 27,200 organisms per c.c. in the water of the Ohio River between September 21, 1898, and January 25, 1899, while the average content of the effluent from the Jewell filter was 400. Data with regard to the operation of mechanical filters are now abundant, since all over the world the operation of these plants is controlled by bacteriological methods. Johnson (1907) has re-

ported some interesting results from the far East. At Osaka, Japan, an average of 200 bacteria per c.c. in the raw water of the Yodo River was reduced, in 1905, to an average of 25 by slow sand filters; at Bethmangala, India, in 1906, mechanical filters treated the water of the Palar River, containing 4350 bacteria per c.c., and yielded an effluent with only 13 per c.c. (Johnson, 1907).

The average monthly results obtained with the mechanical filter plant at Harrisburg, Pa., are included in the table below for comparison with the figures recorded at Washington and Lawrence:

REMOVAL OF BACTERIA BY NATURAL SAND FILTERS AND MECHANICAL FILTERS: BACTERIA PER C.C. IN APPLIED WATER AND EFFLUENT. MONTHLY AVERAGES

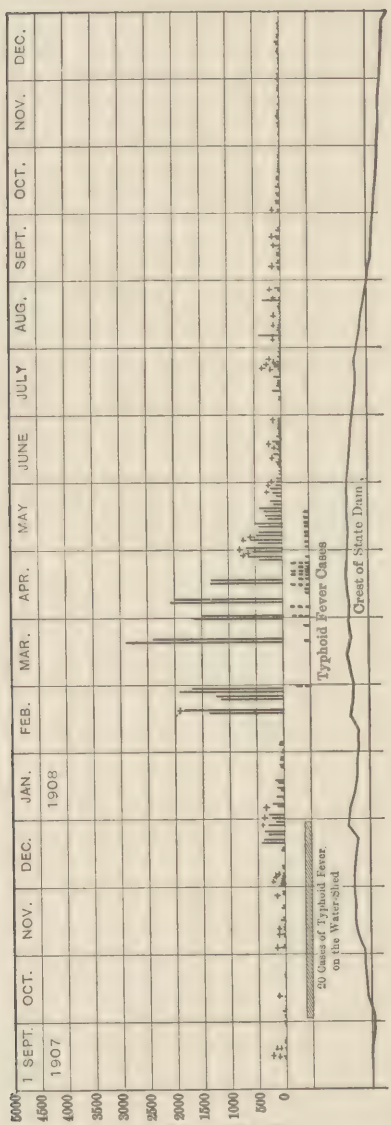
Month	Washington, 1906		Lawrence, 1905		Harrisburg, 1906	
	Applied Water	Effluent	Applied Water	Effluent	Applied Water	Effluent
January	1500	39	14,200	110	9,510	104
February	550	16	14,800	55	21,228	298
March	650	19	10,300	55	31,326	75
April	400	22	3,600	170	39,905	42
May	65	17	1,900	12	6,187	86
June	220	17	9,600	9	2,903	31
July	160	26	3,900	55	685	10
August	190	14	19,500	37	1,637	5
September	130	14	13,500	44	836	12
October	275	16	39,800	110	7,575	63
November	220	12	8,700	70	26,224	236
December	700	45	.....	...	37,525	163

In well-managed purification plants the bacteria in the effluent are determined daily, and any deviation from the normal value at once reveals disturbing factors which may impair the efficiency of the process. In Prussia official regulations demand such systematic examinations and prescribe 50 as the maximum number of bacteria allowable in the filtered water. In the same way the condition of an unpurified surface supply may be determined by daily bacteriological analyses and warnings of danger issued to the public, as has been done at Chicago and other cities. In general, any regular determination of variations from a normal standard furnishes ideal conditions for the bacteriological methods;

and the detection by Shuttleworth (Shuttleworth, 1895) of a break in a conduit under Lake Ontario by a rise in the bacteria of the Toronto water-supply may be cited as a classic example of its application.

The results presented in the diagram on page 44 offer an interesting example of the value of the total count of bacteria as compared with the estimation of *Bact. coli*. This chart, prepared by Mr. J. W. Ackerman, the Engineer of the Auburn, N. Y., Water Board, shows by the vertical lines the total count and by the crosses the presence of colon bacilli in the water supply of Auburn drawn from Lake Owasco. In the year illustrated in this diagram, and in other years, before and after, the total count of bacteria rose sharply at the time of the spring thaw, while colon bacilli were on the whole more abundant during the summer. The explanation of this phenomenon probably is that a certain proportion of colon bacilli are always contributed by the small brooks which enter the reservoir from agricultural land, and which, not being of human origin, have but little significance. At the time of the spring thaws, which for the most part wash an open farming country, the normal contribution of Bact. coli from the fields is obscured by the rain and melting snow, while only the rise in total count registers the fact that contaminating material of all sorts is being washed into the lake. With this contaminating material, for the most part of a harmless nature, human excreta are washed in from certain points on the watershed, and in the spring of 1908 these excreta contained specific typhoid infection and an epidemic in Auburn was the result.

In this case then the total count of bacteria was a more accurate index of danger than the colon content.



Changes in Total Count and B. coli Content in Lake Owasco During the Year of the Water Epidemic of Typhoid Fever at Auburn, N. Y.

(Courtesy of J. Walter Ackerman.)

CHAPTER IV

DETERMINATION OF THE NUMBER OF ORGANISMS DEVELOPING AT THE BODY TEMPERATURE

Relation between Counts Made at 20° and 37°. The count of colonies upon the gelatin plate measures, as we have pointed out, the number of the metatrophic bacteria in general; and the distribution of these forms corresponds with the decomposition of organic matter wherever it may occur. In this great class, there are some species which will grow under a wide variety of conditions. These are present in most waters in small numbers, and in sources contaminated with wash from decaying vegetable matter they occur in abundance. Other metatrophic forms, however, through a semi-parasitic mode of life, have become specially adapted to the peculiar conditions characteristic of the animal body; and these bacteria possess the property of developing most actively at the temperature of the human body, 37° C., which altogether checks the growth of the majority of normal earth and water forms. The determination of the number of organisms growing at the body temperature may throw light, then, on the presence of direct sewage pollution, since the bacteria from the alimentary canal flourish under such conditions, while most of those derived from other sources do not. Savage classifies the bacteria which may be found in water under three headings: normal inhabitants, like *Ps. fluorescens:* unobjectionable aliens (from soil), like *B. mycoides:* and objectionable aliens (from excreta), like *Bact. coli.* The first sort and many of the second sort are generally unable to grow at 37 degrees. This criterion is not an absolute one. Savage (1906) reports an experiment in which unpolluted soil, which had not been manured or cultivated for at least 3 years, was added to tap water, with the result that a 20° count of 76 was increased to 1970, and a 37° count of 3 was raised to 1630. In this case most of the bacteria in the soil were capable of development at body temperature. Experience shows, however, that the numbers of such bacteria which actually reach

natural waters from such sources are seldom large. The count at 37°, therefore, helps to distinguish contamination by wash of the soil of a virgin woodland from pollution by excreta, since in the former case the proportion of blood-temperature organisms is much smaller than in the latter. Furthermore, this method is free from much of the error introduced by the multiplication of bacteria after the collection of a sample, as most of the forms which grow in water during storage cannot endure the higher temperature and consequently do not develop upon incubation. Recently, for example, water from a spring of good quality was shipped to the laboratory from a considerable distance. Gelatin plates showed 4200 bacteria per c.c., but agar plates at 37° were sterile.

Significance of the 37° Count. A majority of the English Committee appointed to consider the standardization of methods for water examination (1904) recommended the body-temperature count as a standard procedure. The American Committee on Standard Methods in its 1905 Report did not recommend this method even for alternative use. In its 1912 report, however, it substituted the 37° for the 20° count, which was dropped out entirely. As we have pointed out in Chapter II, this course seems to us an unwise one, and it was formally condemned at the meeting of the Laboratory Section of the American Public Health Association in September, 1912, by the passage of a vote declaring that "ordinary routine examinations of water for sanitary purposes, and in the control of purification plants for the present, should include the determination of the number of bacteria developing at 20 degrees and 37 degrees." By this action the body-temperature count is properly placed on a par with the 20-degree count as an integral part of sanitary bacteriological water examination.

The body-temperature count must, of course, be made upon agar plates; but otherwise the procedure is much the same as that already described for the routine quantitative bacteriological examination in Chapter II. A beef extract peptone medium containing 1.2 per cent of agar is specified by the Standard Methods Committee in its recent report. The agar used should be dried at 105° C. for 30 minutes, as commercial agar itself contains more or less water.

The period of incubation ordinarily adopted for body-temperature counts is 24 hours. Lederer and Bachmann (1911) find that

with sewage effluents a 48-hour period at 37° counts may yield counts from two to six times as high as those obtained in 24 hours; it is questionable, however, whether the higher counts thus given would compensate for the loss of time. The adoption of a 24-hour period by the Standard Methods Committee in any case represents an almost universal practice.

In using agar plates at 37° difficulty is sometimes caused by the spreading of colonies of certain organisms over the surface of the plate in the water of condensation which gathers; this may be avoided by inverting the plates after the agar is once well set, or still better by the use of plates provided with earthenware tops, as suggested by Hill. The porous earthenware absorbs the water which condenses on it, the surface of the plate remains comparatively dry, and the percentage of "spread" plates is reduced from 30 per cent to 1 per cent (Hill, 1904). Special pains must be taken, however, to keep the atmosphere in the incubator nearly saturated with moisture or errors will be introduced by the excessive evaporation of the medium used.

Use of Litmus Lactose Agar. Additional evidence as to the character of a water sample may be obtained with little extra trouble by adding a sugar and some sterile litmus to the agar medium and observing the fermenting powers of the organisms present, as first suggested by Wurtz (Wurtz, 1892) for the separation of *Bact. coli* from *Bact. typhosum*. It happens that the most abundant intestinal organisms, belonging to the groups of the colon bacilli and the streptococci, decompose dextrose and lactose with the formation of a large excess of acid. The decomposition of the latter sugar, is, on the other hand, almost entirely wanting among the commoner saprophytic bacteria, and therefore lactose is most commonly used in making sugar agar, 1 per cent being added to the medium just before sterilization. In pouring the plate a cubic centimeter of sterile litmus or azolitmin solution should be added. After incubation the colonies of the acid-forming organisms will be clearly picked out by the reddening of the adjacent agar. Only those colonies which are sharply colored should be considered as significant, since certain bacteria of the hay-bacillus group produce weak acid and faint coloring of the litmus.

When polluted waters are examined in this manner the number of organisms developing on the lactose-agar plate will be very

high, almost equalling in some cases the total count obtained on gelatin. Chick (Chick, 1901), using a lactose-agar medium with the addition of one-thousandth part of phenol, found, of colon bacilli alone, 6100 per c.c. in the Manchester ship canal; 55–190 in the polluted River Severn, and numbers up to 65,000 per gram in roadside mud. In an examination of water from the Charles River above Boston, 37° counts ranging from 9800 to 16,900 have been found. The average result of 56 examinations of Boston sewage from July to December, 1903, showed 5,430,000 bacteria per c.c. at 20°, and 3,760,000 per c.c. at 37°, of which 1,670,000 were acid formers. The average of 25 samples examined in July and August, 1904, showed 1,690,000 bacteria per c.c. at 20° and 1,400,000 at 37°; 429,000 per c.c. were acid formers (Winslow, 1905).

In unpolluted waters not only is the absolute number of organisms developing at the body temperature less, but its ratio to the gelatin count is very different. Rideal (Rideal, 1902) states that the proportion between the two counts in the case of a London water in a year's examination was on the average one to twelve. Mathews (Mathews, 1893) in 1893 gave the following figures, the contrast between the ponds and streams, which were presumably exposed to pollution, on the one hand, and the wells, springs, and taps, on the other, being marked.

Source of Water	Average Number of Colonies per c.c.	
	Gelatin, 20°	Wurtz Agar, 37.5°
Wells, springs	1664	28
Reservoirs	153	43
Ponds	296	95
Taps	242	24
Streams	273	101

According to the English Committee appointed to consider the Standardization of Methods for the Bacterioscopic Examination of Water (1904), the ratio of the 20° count to the 37° count in good waters is generally considerably higher than 10 to 1. "With a polluted water this ratio is approached, and frequently becomes 10 to 2, 10 to 3 or even less." The relation between the two

RELATION OF 20° AND 37° COUNTS IN SAMPLES OF WATER FROM APPARENTLY UNPOLLUTED SOURCES

(Winslow and Nibecker, 1903)

Source of Samples	Number of Samples	Gelatin Plates, 20°	Litmus-lactose-agar Plates 37°		Dextrose-Broth Tubes			
		Average Number of Colonies	Average Number of Colonies	Plates Showing Red Colonies	Number of Tubes	Number of Tubes with Gas	Number of Tubes with Gas 1-0	Number of Tubes with Gas 2-1
Cambridge supply (tap)	5	94	11	0	15	0	0	0
Wakefield and Stoneham supply (tap)	7	59	6	0	21	0	0	0
Lynn supply (tap)	6	16	3	0	18	0	0	0
Brookline supply (tap)	1	....	18	2	3	0	0	0
Plymouth supply (tap)	6	35	2	0	18	0	0	0
Peabody supply (tap)	3	141	21	0	9	2	2	2
Dedham supply (tap)	6	3717	1	0	18	0	0	0
Newburyport supply (tap)	6	36	9	0	18	0	0	0
Salem supply (tap)	5	232	14	0	15	0	0	0
Taunton supply (tap)	4	13	2	0	12	0	0	0
Sharon (well) (tap)	3	738	46	2	9	3	0	3
Medford supply (tap)	5	524	8	0	15	0	0	0
Milton supply (tap)	2	4700	0	0	6	0	0	0
Westerly, R. I., supply (tap)	1	....	12	0	3	0	0	0
Brooks	61	223	7	0	183	13	13	0
Driven wells	15	18	1	0	45	0	0	0
Springs	32	294	2	0	95	13	13	0
Ponds fed by brooks	15	167	9	0	45	1	1	1
Melted snow	1	....	4	0	3	0	0	0
Pools in fields	22	365	31	0	66	2	2	0
Pools in woods	22	181	3	0	65	0	0	0
Roadside pools	10	811	4	0	30	2	2	2
Stream, Blue Hill Reservation	1	....	0	0	3	0	0	0
Flow from rocks	2	47	0	0	6	0	0	0
Ponds fed by springs	6	188	2	0	18	0	0	0
Drainage from manured pasture	1	1235	27	0	3	0	0	0
Swamps	3	269	6	0	9	5	5	0
Rain-water after twelve hours' heavy fall	7	....	2	0	21	0	0	0
Shallow well in Lynn woods	1	15	1	0	3	0	0	0
Totals	259	....	..	4	775	41	38	3

counts does not, however, always work out quite so neatly. Tanner (1916) in a series of 4379 water samples found 412 which gave higher numbers on agar at 37° than on gelatin at 20°, most of them being shallow wells or treated waters. The other 3967 samples showed an average ratio of the 20° count to the 37° count of 2.4, the ratio being low for wells and treated waters and high for surface waters, just the reverse of what offhand would have been expected.

In 1903 Nibecker and one of ourselves (Winslow and Nibecker, 1903) made an examination of 259 samples of water from presumably unpolluted sources in Eastern Massachusetts, including public supplies, brooks, springs, ponds, driven wells, and pools in the fields and woods, with a view to testing the value of the body-temperature examination. In many cases the samples showed high gelatin counts, since some of the waters were exposed to surface wash from vacant land, but the average number of organisms developing on lactose agar at 37 degrees was less than 8 per c.c., as will be seen by reference to the table on the following page. The highest individual counts obtained were 95 in a meadow pool, 83 in a brook, and 74 in a barnyard well, the latter probably actually polluted. Only two samples in the whole series, one from the well above mentioned, gave any red colonies on the agar plates.

BACTERIAL CONTENT OF 147 SHALLOW WELLS

Percentage of Samples in Each Group

Bact. coli		Bacteria per c.c. 0	1–10	11–20	21–50	51–100	101–500	501–1000	1001–2000	2001–3000
−	Gelatin, 20°	3	16	14	16	11	31	5	4	
+		..	..	5	..	10	57	10	14	5
−	Agar, 37°	15	63	10	10	1	1			
+		..	31	35	22	4	4	4		
−	Red colonies	86	12	2						
+		30	52	9	9					

For a series of shallow surface wells recently examined by one of us (S. C. P.) a similar relation is indicated in the table above;

124 samples which showed no colon bacilli and were apparently unpolluted, gave an average of 190 bacteria per c.c. at 20° and 8 at 37° with less than one red colony per c.c.; 23 samples which did contain colon bacilli averaged 570 bacteria per c.c. at 20° and 55 at 37° with an average of 7 red colonies.

AVERAGE NUMBER OF BACTERIA AND ACID-PRODUCERS DEVELOPING AT 20°, 40°, AND 50° C., WITH DIFFERENT CLASSES OF WATERS

GAGE (1906). REARRANGED

	Bacteria per c.c.			Acid-producing Bacteria		
	20° C. 4 D.	40° C. 24 Hrs.	50° C. 24 Hrs.	20° C. 4 D.	40° C. 24 Hrs.	50° C. 24 Hrs.
Sewage	2,990,000	557,500	7,700	1,940,000	346,000	4,400
"	1,676,000	360,000	29,500	1,032,000	283,000	24,900
Septic effluent	485,000	126,500	410	241,000	90,000	240
Contact effluent	146,600	26,100	8,300	112,400	22,700	8,000
" "	389,000	59,300	8,000	292,000	45,000	8,000
" "	306,000	89,600	485	193,000	46,000	200
Trickling filter effluent	15,500	1,730	154	15,200	1,360	100
Do	23,300	2,030	54	16,000	1,180	20
Canal water	16,400	112	5	6,700	87	2
River water	16,900	207	4	2,500	134	2
Settled canal water	2,800	212	2	1,650	66	1
Sand filter effluent (sewage)	1,640	1,375	2	2,360	1,195	1
Do	35	4	0	29	2	0
Do	1,300	130	1	345	119	0
Do	670	170	2	1,045	154	0
Water filter effluent	32	3	1	6	1	1
Do	715	170	2	259	101	1
Do	62	1	0	16	0	0
Do	150	22	1	14	17	1
Do	64	5	1	11	3	1
Shallow well	1,000	2	0	3	1	0
" "	507	72	0	82	55	0
Pond	27	1	0	8	1	0
"	71	8	0	30	5	0
Spring	49	0	0	6	0	0
"	80	2	0	8	2	0
Driven well	41	0	0	0	0	0

Significance of High Temperature Counts. Important data as to the distribution of bacteria which will develop at high temperatures may be found in a paper by Gage (1906), coupled with

a suggestive discussion of the general significance of bacterial ratios. The table on page 51 shows some of the most significant results obtained by plating waters of various degrees of purity at 20°, 40° and 50°. We have rearranged the lines of the table so as to make the progression from more to less polluted waters a fairly regular one. The colony count at 50° shows an even sharper differentiation than that at 40°. Gage rightly concludes that "the information to be obtained by counts of bacteria and acid-producing organisms at any one of the above temperatures is greatly increased by the combination of the results obtained from counts at two or more temperatures."

In warm weather the interpretation of the body-temperature count must be made less rigid than at other seasons. Recent investigations have shown that in midsummer bacteria capable of growth at 37° are more abundant in normal waters than in winter and spring. Race (1916) points out that the ratio of the 37° count to the 20° count varies directly with the outdoor temperature to which the natural waters are subjected, the ratio being high in summer and fall and decreasing in winter and spring.

20° AND 37° COUNTS OF RAW WATER AT WILMINGTON FILTER PLANT

(WHIPPLE, 1913)

Month	1908			1909		
	Bacteria per c.c.		Per Cent	Bacteria per c.c.		Per Cent
	Gelatin, 20°	Bile-agar, 37°		Gelatin, 20°	Bile-agar 37°	
January	4630	124	2.7	3880	94	2.3
February	6830	358	5.3	4800	260	5.4
March	8800	350	4.0	4620	387	8.4
April	3170	149	4.7	5080	347	6.7
May	2010	119	5.9	3340	229	6.8
June	1640	241	14.7	2350	158	6.7
July	3150	432	13.7	2940	57	1.9
August	3140	451	14.4	1430	230	16.1
September	3400	644	18.9	2620	619	22.8
October	5180	439	8.5	1380	129	9.4
November	6850	78	1.2	1650	97	3.9
December	4100	203	4.9	4150	194	4.7

Winslow and Phelps examined 86 samples from springs, wells, brooks and pools during the winter and spring months and found only 12 which showed more than 25 bacteria per c.c. and only 3 which showed more than 100 per c.c. on lactose-agar. On the other hand, of 58 samples from corresponding sources examined in summer, 16 contained more than 100 bacteria per c.c. A series of 20 pools, ponds, and brooks at Mt. Desert, Me., which were entirely free from human or animal pollution, were examined in the late summer of 1906. Only 4 of the 20 samples gave counts under 25 at 37°, and 7 of them gave counts over 100, the highest figure being 425.

Whipple (1913) gives some figures for the raw water at the Wilmington, Del., filter plant (page 52) which bring out the seasonal variation very clearly.

COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, MERRIMAC RIVER WATER, AT LAWRENCE, MASS.

(Clark and Gage, 1909)

Sample	Bacteria per c.c.			
	Untreated Water		Treated Water	
	20°	37°	20°	37°
A	3,400	30	12	4
B	28,900	130	4	4
C	14,000	75	35	47
D	3,700	81	43	62

Another special case in which the ratio between the 20° and the 37° count fails to be significant is that of a water which has been treated with bleaching powder. Most of the bacteria which survive chlorine treatment are of course spore formers, many of them belonging to the hay bacillus group, and it happens that most of these spore formers can grow at body temperature. Thus it is common to get counts as high at 37° as at 20° with such waters, although the absolute numbers are generally small. This point is illustrated in the two tables below, showing the results of experimental treatment of Merrimac River water at the Lawrence

Experiment Station and of swimming pool water at the University of Wisconsin.

The organisms found in water after efficient chlorination will of course in general be of innocuous types except in the possible case of the presence of *Bact. welchii* forms. Smeeton (1917) examined 105 organisms isolated on aerobic plates from chlorine disinfected water and found that 89 were aerobic spore formers (*Bact. subtilis* being most abundant), 11 chromogenic non-spore-forming water bacteria and 5 yellow cocci.

COMPARATIVE EFFECTS OF CHLORINE DISINFECTION UPON 20° AND 37° COUNTS, SWIMMING POOL WATER AT UNIVERSITY OF WISCONSIN

(Tully, 1912)

Sample	Bacteria per c.c.			
	Untreated Water		Treated Water	
	20°	37°	20°	37°
A	275	16	0	1
B	445	480	4	5
C	920	483	8	8
D	5,630	680	4	2
E	19,100	1,140	30	45
F	24,000	1,190	130	120
G	10,000	1,080	14	27
H	1,700	690	15	9
I	2,570	780	12	30
J	2,800	560	27	66

Under ordinary conditions it is clear that organisms growing at the body temperature and those fermenting lactose are not numerous in normal waters. The absolute count at 37° seldom exceeds 50, and is rarely over 10 per cent of the 20° count, except after hot periods in the late summer; acid producers are generally entirely absent. On the other hand, the numbers on the litmus-lactose-agar plate will be likely to run into hundreds with a good proportion of red colonies when polluted waters are examined.

CHAPTER V

THE COLON GROUP OF BACILLI AND METHODS FOR THEIR ISOLATION

The Colon Group of Bacilli. The *Bacterium coli* was first isolated by Escherich (Escherich, 1884) from the fæces of a cholera patient. It was subsequently found to be a normal inhabitant of the intestinal tract of man and many other animals, and to occur regularly in their excreta, and on this account it became of the highest interest and importance to sanitarians, since its presence in water-supplies was regarded as direct evidence of sewage pollution.

Specific disease germs are difficult to isolate even when many are present; and water may of course be grossly polluted with sewage without any specific disease germs being present at all. All sewage-polluted water, however, is potentially dangerous, since where fæcal matter exists, disease germs are at any time likely to appear. A test for fæcal material as distinguished from infected material is, therefore, essential; and for such a test the colon group of bacilli are specially well suited. They are not dangerous in themselves, but they are significant as indices of the probable presence of disease germs.

The so-called colon bacillus is a member of a considerable group of allied organisms which constitute the genus, *Bacterium* (Ehrenberg); and this genus is defined by the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (1920) in the following terms.

> "Gram negative, evenly staining rods. Often motile, with peritrichic flagella. Easily cultivable, forming grape-vine leaf or convex whitish surface colonies. Liquefy gelatin rarely. All forms except *Bact. alcaligenes* and the *Bact. abortus* group attack the hexoses and most species ferment a large series of carbohydrates. Acid formed by all, gas (CO_2 and H_2) only by one series. Typically intestinal

parasites of man and the higher animals although several species may occur on plants and one (*Bact. aerogenes*) is widely distributed in nature. Many species pathogenic."

The genus, *Bacterium*, belongs to the family Bacteriacea, and hence its members form no spores.

The commonest members of the genus *Bacterium* are the organisms which belong to the colon-typhoid series, a collection of bacteria which are clearly allied to each other in certain respects but which differ widely in their power to attack carbohydrates. They form a gradation from *Bact. alcaligenes* which possesses almost no fermentative powers at one end to *Bact. aerogenes*, which vigorously attacks a large variety of carbohydrates, at the other. The general relations of the more important species are indicated in the table below (Winslow, Kligler and Rothberg, 1919).

Species	Hexoses	Maltose	Mannitol	Xylose	Arabinose	Rhamnose	Sorbitol	Dulcitol	Lactose	Salicin	Sucrose	Raffinose	Inosite	Dextrin	Gas	Voges-Proskauer	Methyl red	Milk	Gelatin	Indol	Lead Acetate	Motility	Pathogenic
Bact. aerogenes	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Coag.	−	−	−	−	−
Bact. cloacae	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	Coag.	+	−	−	+	−
Bact. neapolitanus	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	−	+	Coag.	−	+	−	+	−
Bact. communior	+	+	+	+	+	+	+	+	+	−	+	+	−	−	+	−	+	Coag.	−	+	−	+	−
Bact. coli	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+	Coag.	−	+	−	+	−
Bact. acidi-lactici	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	+	Coag.	−	+	−	+	−
Bact. morgani	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	al.	−	+	+	+	+
Bact. schottmulleri	+	+	+	+	+	+	+	+	−	−	−	−	+	−	+	−	+	ac. al.	−	−	+	+	+
Bact. enteritidis	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	ac. al.	−	−	+	+	+
Bact. suipestifer	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	+	ac. al.	−	−	−	+	+
Bact. gallinarium	+	+	+	+	+	+	+	+	−	−	−	−	−	+	−	−	+	ac. al.	−	−	−	−	+
Bact. pullorum	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	+	ac. al.	−	−	−	−	+
Bact. paratyphosus	+	+	+	−	+	+	+	+	−	−	−	−	−	−	+	−	+	ac. al.	−	−	−	+	+
Bact. typhosus	+	+	+	+	−	−	+	−	−	−	−	−	−	+	−	−	+	ac. al.	−	−	+	+	+
Bact. dysenteriae	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	ac. al.	−	+	−	−	+
Bact. shigae	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	ac. al.	−	−	−	−	+
Bact. alcaligenes	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	al.	−	−	+	+	−

It will be noted that the true pathogenic members of this series, the dysentery, typhoid and paratyphoid organisms, all fail to ferment lactose. On the other hand the six species at the top of the table do ferment lactose with the production of gas. These lactose-fermenters are the characteristic organisms of the intestine and, since it is a test for excremental pollution which we need this

group, commonly called the colon group, is generally used as the most satisfactory index of water pollution.

The commonly used tests for water make no clear distinction between the individual members of the colon group (the six first species listed in the table on page 56). The group may be divided, however, into two main sections, the *Bact. aerogenes* section and the *Bact. coli* section whose relative importance is probably very different. We shall return to this question in a later paragraph; but it will be well to present here a somewhat more detailed classification of the colon group taken from a monograph by Levine (1921) which indicates clearly the relations of the species in question.

Classification of the Colon Group

(Levine, 1921)

(Group includes all non-sporing, Gram-negative, short rods, fermenting glucose and lactose with acid and gas production and growing aerobically.)

I. Producing acetyl-methyl-carbinol (Voges-Proskauer reaction positive); alkaline to methyl-red and forms two or three times as much carbon dioxide as hydrogen from glucose. Capable of utilizing uric acid as a source of nitrogen. *Bact. aerogenes* section.

1. Glycerol and starch fermented with acid and gas formation; non-motile, gelatin not liquefied. *Bact. aerogenes.*

2. Glycerol and starch not fermented; motile, gelatin liquefied. *Bact. cloacae.*

II. Not producing acetyl-methyl-carbinol (Voges-Proskauer reaction negative); acid to methyl-red, and carbon dioxide and hydrogen produced in approximately equal volumes from glucose. Cannot utilize uric acid as a source of nitrogen. *Bact. coli* section.

1. Sucrose fermented with acid and gas.
   A. Non-motile.
      a. Salicin fermented with acid and gas. *Bact. neapolitanum.*
      b. Salicin not fermented. *Bact. coscoroba.*
   B. Motile. *Bact. communior.*

2. Sucrose not attacked.
   A. Salicin fermented with acid and gas. *Bact. coli.*
   B. Salicin not attacked. *Bact. acidi-lactici.*

It will be noted that Levine's classification differs from the one cited on page 56 in separating *Bact. communior* from *Bact. neapolitanum* on the basis of motility (which Winslow, Kligler and Rothberg regard as an inconstant character) rather than by salicin fermentation; and in recognizing another species, the non-motile, salicin-negative *Bact. coscoroba*. These differences are admittedly only of minor importance; but the differentiation between the *Bact. coli* section and the *Bact. aerogenes* section has a fundamental significance.

Distinction between Bact. coli and Bact. aerogenes. We owe the recognition of the importance of the difference between *Bact. coli* and *Bact. aerogenes* primarily to Rogers and Clark and their associates in the Department of Agriculture at Washington. These observers (Rogers, Clark and Davis, 1914) discovered the fact that when the gas produced by colon-like organisms in dextrose broth is collected under a vacuum and analyzed with proper precautions, two groups may be clearly distinguished by the ratio of hydrogen to carbon dioxide produced. One well-defined group, *Bact. coli*, gave a constant ratio of about one part of carbon dioxide to one part of hydrogen, while the rest gave a ratio of over 1.4 to 1.0 and usually 2 to 1 or more. The high-ratio group was usually saccharose-positive, dulcite-negative. The low-ratio group included strains of more widely differing reactions.

In a subsequent study, Rogers, Clark and Evans (1914) present a most significant comparison of the types of colon bacilli occurring in milk and in bovine fæces, respectively. Of 125 strains isolated from milk, about half belonged to the high-ratio group defined above (presumably corresponding to *Bact. aerogenes*), while in 150 cultures from bovine fæces this high-ratio group was found only once. The same workers (Rogers, Clark and Evans, 1915) studied 166 strains of colon-like bacteria isolated from grains and found that only 8 of them gave the characteristic gas ratio of *Bact. coli*, 7 forming hydrogen only and 151 showing the high gas ratio of the *Bact. aerogenes* type. The work of Clark and Lubs (1915) then demonstrated that the high and low ratio groups (*Bact. aerogenes* and *Bact. coli*, respectively) were also differentiated by the hydrogen ion concentration produced by them in suitable culture media and that this difference in hydrogen ion concentration could be observed in a very simple fashion by incubation in a peptone-glucose-phosphate medium and testing

with methyl red, the colon bacilli giving an acid reaction, the *Bact. aerogenes* type an alkaline one.

The next important step in this field was the demonstration, largely due to Levine (1916[a]) of a close correlation between the Voges-Proskauer test and the methyl red reaction which confirmed the view that organisms of the *Bact. aerogenes* group (methyl-red negative, Voges-Proskauer positive, high gas ratio) are relatively rare in fæces of men and animals. He believes that organisms of this type are presumably normal inhabitants of the soil (Levine, 1916[b]; 1918).

The Voges and Proskauer reaction has been extensively used by MacConkey and his followers in England and by Bergey and Deehan (1908) in this country. After the carbon dioxide in the fermentation tube has been absorbed by caustic soda, if the tube be allowed to stand, an eosin-like color gradually develops in the open arm, due to the presence of acetyl-methyl-carbinol. West (1909) points out that the test used by Rivas, the boiling of 1 to 4 c.c. of a 48-hour dextrose broth culture with 5 c.c. of a 10 per cent caustic soda solution, is a quick method of obtaining the Voges and Proskauer reaction. A yellow color is produced under these conditions by the sugar alone, and a pinkish eosin-like color when the acetyl-methyl-carbinol is present. The reaction is hastened by shaking or blowing into the tube to promote oxidation. West confirms the early conclusion of MacConkey and Bergey that this reaction is characteristic of the *Bact. aerogenes* and *Bact. cloacæ* types (saccharose positive, dulcite negative organisms).

The 1923 Standard Methods still recommend that the Voges-Proskauer test should be made by adding 5 c.c. of a 10 per cent solution of potassium hydroxide to 5 c.c. of a 5-day dextrose-broth culture and holding over night for the appearance of the eosin pink color.

Bunker, Tucker and Green (1918) recommend what appears to be a valuable modification of the Voges-Proskauer technique (first suggested by Clemesha) which involves the addition to 1 c.c. of culture in a watch glass of 0.5 c.c. of 45% NaOH. Under such conditions the characteristic color which appears in media incubated for 2 days at 30° C., develops in an hour and a half and the reaction develops in a much larger proportion of cases. Chen and Rettger (1920) recommend that 5 to 6 c.c. of the culture be added to an equal volume of 10 per cent KOH in a test tube, well

shaken, and incubated at 30° C. for one to three hours, after which the liquid is again shaken vigorously until it becomes foamy before reading. They also confirm the finding that prolonged incubation of the culture before making the test is quite unnecessary.

The physiological reasons for the differences observed between the end products of the colon and aerogenes types have been discussed in detail by Burton and Rettger (1917) who believe that the two types of fermentation, — one producing small amounts of hydrogen, large amounts of CO_2 and the butylene glycol which after oxidation gives the Voges-Proskauer reaction, the other producing equal amounts of CO_2 and hydrogen with little or no butylene glycol, — are quite independent of each other.

On the whole, however, the most satisfactory explanation of the phenomena is that offered by Ayers and Rupp (1918) who believe that in a simple synthetic medium both *Bact. coli* and *Bact. aerogenes* ferment dextrose in a similar fashion, the organic acids formed reacting with the phosphate, forming salts of organic acids and acid phosphates. In the case of both *Bact. aerogenes* and *Bact. coli* the fermentation of the sugar and of the organic acid salts then proceeds simultaneously and the difference in end products is due merely to a difference in the rate at which these two processes go on in the case of the two organisms.

Koser (1918; see also Koser and Rettger, 1919) added a new differential test of considerable value by showing that *Bact. aerogenes* will grow in a special synthetic medium containing uric acid as the sole source of nitrogen while organisms of the *Bact. coli* type are unable to do so.

It should be remembered, of course, that such correlations as those discussed are never absolutely constant among the bacteria. Perry and Monfort (1921) review the literature on this point and describe a number of strains isolated by themselves which show aberrant combinations of the methyl-red, Voges-Proskauer and uric acid reactions. It has been made clear by numerous investigations that sudden mutations are likely to occur in a pure strain of bacteria, particularly in regard to the fermentation of carbohydrates. Twort (1907) reports that by continued cultivation in sugar media he was able to develop fermentative power in certain members of the Gärtner group which lacked such powers before.

The work of Massini, Müller, Sauerbeck, Konrich and others (well summarized by Konrich, 1910) has also shown that mutations

capable of fermenting sugars may suddenly arise from a parent strain lacking this power. Burri (1910) has contributed to the same problem and has found that the latent power to ferment a given sugar is released by growing the organism on that particular sugar, but that, as Konrich and the others show, only a certain proportion of the cells develop this power. The most important recent studies of bacterial mutation have been made by Penfold. In his latest communication (Penfold, 1912) he shows that many bacteria of the colon-typhoid group produce a mutant capable of fermenting lactose, that all strains of the typhoid bacillus produce dulcite and iso-dulcite mutants, that many paratyphoid and Gärtner group bacilli produce raffinose mutants, and that other mutations also occur. The general phenomena are the same in each case. A strain which normally fails to ferment a given carbohydrate is grown upon a solid medium containing that carbohydrate. As the colonies develop there appear upon them raised papillæ of a different consistency from the rest of the colony and colored red if litmus be present. Transplants from the papillæ give pure cultures of a strain fermenting the carbohydrate in question and forming no papillæ. Transplants from the other portions of the colony give the original strain, non-fermenting, but capable of producing fermenting mutants as before. The identity of derivative strain in all other respects has been made clear by exhaustive cultural tests and serum reactions; and Penfold has shown that the whole process may be repeated, starting from an isolated single cell. Similar results have been very recently reported by Bergstrand (1923).

The Isolation of Bacteria of the Colon Group from Water. We shall return later to a consideration of the differences between the *Bact. coli* and *Bact. aerogenes* sections of the colon group and shall consider for the present the principles involved in the isolation of the group as a whole (non-spore-forming, aerobic Gram-negative bacilli, fermenting dextrose and lactose with acid and gas production).

The most obvious and distinctive characteristic of this group is the fermentation of lactose with the production of acid and gas and both these products have been used for its ready identification. The litmus-lactose-agar plate, discussed above in Chapter IV, furnishes one ready method for the isolation of *Bact. coli* from water, and it was used by Sedgwick and Mathews for

the purpose as early as 1892 (Mathews, 1893). If plates are made with agar containing both lactose and litmus, the colon colonies develop as red spots in a blue field. Since other organisms (notably the streptococci) may also develop red colonies, it is necessary to examine them further. This is done by fishing from isolated colonies, replating and inoculating into other media for identification.

The plate method of isolation was recommended by the Committee on Standard Methods of Water Analysis (1912) for sewages and polluted waters, in which colon bacilli are present in 1 c.c. or less. They suggested that Petri dishes with porous covers be used and that incubation be carried out at 40° instead of 37°. For success in the use of this method it is necessary to get a sufficient dilution so that colonies may be well isolated, and to this end it is advisable that a number of different dilutions be employed, a series of plates being prepared from each. Under any conditions the detection of the colon bacillus is seriously hampered by the development of other forms.

The test for the colon bacillus may be made more delicate by a preliminary cultivation of the sample in a liquid medium for 24 hours at 37°, thus greatly increasing the proportion of these organisms present before plating. As suggested in the classic researches of Theobald Smith (Smith, 1892), this method may be made approximately quantitative by the inoculation of a series of tubes with measured portions of the water. If, for example, of ten tubes inoculated each with $\frac{1}{100}$ of a cubic centimeter, four show *Bact. coli*, we may assume that some 40 of these organisms were present in the cubic centimeter. Irons (Irons, 1901), in a comparative study of various methods for the isolation of *Bact. coli*, was one of the first to show that the preliminary enrichment frequently gave positive results when the results of the direct use of the agar plate were negative.

The medium most commonly used in the United States prior to 1906 for preliminary enrichment was ordinary broth to which 1.0 per cent of dextrose had been added, and the reaction brought to the neutral point. Into each of a number of fermentation-tubes of this medium a measured quantity of the water to be examined was inoculated, and the culture incubated for 24 hours at 37.5° C. It used to be customary to incubate for 48 hours. Experience has, however, shown that a 24-hour period gives

approximately the same results if the production of gas rather than any specified amount of gas is the criterion of a positive test. Longley and Baton (1907) found that of 1901 enrichment tubes giving positive tests after 48 hours only 173 showed no gas in 24 hours; of these latter only two contained *Bact. coli.* At the end of 24 hours and again after 48 hours, the tubes should be examined for gas formation. If gas is found, a small amount of the culture should be added, after proper dilution, to a suitable solid medium for isolation of the organism present.

With polluted waters it will be found advantageous to plate out on the first appearance of gas (4–8 hours). It has been shown by one of us (Prescott, 1902[b]) that a very rapid development of *Bact. coli* takes place in the first few hours after dextrose solutions are inoculated with intestinal material, and a nearly pure growth of colon bacilli often results, while other bacteria multiply more slowly. With highly polluted waters gas formation will probably begin within 12 hours, but with fewer colon bacilli present the duration must be increased. If the period of incubation be too long continued, trouble in the subsequent steps of the isolation may be encountered because of overgrowths by the sewage streptococci, or other forms which check the growth of the colon bacilli in the later stages of fermentation and finally kill them out. Even with pure cultures of colon bacilli Clemesha (1912[b]) has shown that sugar-broth tubes may be almost sterile after 4 days.

When it is desired to examine samples larger than 10 c.c. it becomes necessary to modify the enrichment process by adding the nutrient material to the water instead of the reverse. For this purpose sugar broth may be added to the sample of water to be enriched as suggested by Gage (Gage, 1901). Generally 10 c.c. of the broth is added to 100 c.c. of the water. The sample is then incubated at 37° for 24 hours, and if at the end of that time growth has taken place, a cubic centimeter is inoculated into a dextrose tube.

The Presumptive Test for Bact. Coli. Experience with the dextrose broth fermentation tube as a first step in the isolation of colon bacilli soon led to the conclusion that a fair idea of the sanitary quality of water could be obtained from the results of this test taken by themselves and without the further process of isolating specific cultures. It appeared that a rather definite propor-

tion of tubes showing a characteristic fermentation proved on further examination to contain bacilli of the colon group; and it was therefore suggested that the dextrose broth test alone might be used as a rapid " presumptive " test.

In earlier years, Irons (Irons, 1901) was perhaps the first to call attention to the value of this method, stating that " when the dextrose tube yields approximately 33 per cent of CO_2, Bacillus coli communis is almost invariably present." In the next year the reliability of the fermentation test as an indication of *Bact. coli* was worked out by Gage (Gage, 1902) as given in the table below:

	1 c.c.	100 c.c.
Number of samples tested	5172	1375
Number giving preliminary fermentation	1036	474
Per cent of latter proved to contain coli	70	71

Whipple (Whipple, 1903) examined a large number of surface-water supplies by this " presumptive test " and obtained striking results, shown in the following table. The waters are arranged in six groups according to the results of sanitary inspection, group I including waters collected from almost uninhabited watersheds, and group VI waters too much polluted to be safely used for domestic purposes.

PERCENTAGE OF SAMPLES OF WATERS OF VARIOUS SANITARY GRADES GIVING POSITIVE TESTS FOR BACT. COLI WHEN DIFFERENT AMOUNTS WERE EXAMINED

(Whipple, 1903)

Group	0.1 c.c.	1.0 c.c.	10 c.c.	100 c.c.	500 c.c.
I	0.0	3.5	20.8	50.0	50.0
II	5.0	7.3	15.0	60.0	60.0
III	0.0	7.0	50.0	50.0	60.0
IV	4.0	6.8	41.7	67.0	75.0
V	5.0	13.0	75.0	100.0	100.0
VI	5.0	20.2	75.0	80.0	100.0

In view of these results Whipple suggested the following provisional scheme of interpretation:

Sanitary Quality	Presumptive Test for Bact. coli				
	0.01 c.c.	0.1 c.c.	1.0 c.c.	10.0 c.c.	100 c.c.
Safe	0	0	0	0	+
Reasonably safe	0	0	0	+	+
Questionable	0	0	+	+	+
Probably unsafe	0	+	+	+	+
Unsafe	+	+	+	+	+

It is undoubtedly true that a negative presumptive test is generally obtained with unpolluted waters. For example, in a study previously cited, Winslow and Nibecker (1903) reported that of 775 dextrose-broth tubes inoculated from 259 unpolluted sources only 41 showed gas. On the other hand, it is equally true that in a large proportion of cases colon bacilli are isolated from positive dextrose-broth tubes. Longley and Baton (1907) in the examination of 3553 samples of Potomac water obtained positive tests 794 times, while *Bact. coli* was actually present 529 times; 67 per cent of the presumptive tests were therefore correct. Gage (1902), in the Massachusetts work cited above, found that 70 per cent of his fermented dextrose tubes contained *Bact. coli.*

The work of recent years has made it clear, however, that the general coincidence of positive presumptive tests with the presence of *Bact. coli,* is open to disastrous exceptions.

According to Clark and Gage (1903) there are 58 well-described species of bacteria which give the presumptive test in dextrose-broth, of which 23 are widely separated from the *Bact. coli* group. An unpublished investigation by Winslow and Phelps indicates that the result of the dextrose broth test is markedly influenced by the factor of temperature. Their work consisted in the examination of 185 samples of water from 90 different sources, ponds, brooks, pools, wells and springs in five different States, Maine, New Hampshire, Massachusetts, Michigan and Virginia, at three different seasons of the year. All the waters examined were, as far as could be determined, free from specific pollution, although washings from roads or pastureland might have had access to some of them. Most of the sources were undoubtedly unpolluted and the examination of 119 samples for *Bact. coli* yielded only 12 positive results. The presumptive test, however, was obtained in a

large proportion of the cases, and much more often in summer than in winter or spring, as indicated in the table below.

DEXTROSE BROTH FERMENTATION IN 185 SAMPLES OF NORMAL WATERS AT DIFFERENT SEASONS

(Winslow and Phelps)

Percentage of Positive Results

	Summer, 1906	Winter	Spring	Summer, 1907
Framingham, Mass.....	87	62	23	57
Ann Arbor, Mich.....	95	47	..	..
Exeter, N. H.....	82	10	44	50
Richmond, Va.....	..	14	14	..
Mt. Desert, Me.....	95	..	..	..
All stations.....	91	37	25	54

The Ann Arbor waters in this series included a number of driven wells, and the Mt. Desert sources were mountain brooks and ponds of the highest sanitary quality.

Phelps and Hammond (1909) in this country, Fromme (1910) in Germany, Houston (1911) in England and Clemesha (1912[a], 1912[b]) in India have all reported similar results which make it clear that a type of organisms fermenting dextrose but not lactose is fairly abundant in stored and relatively pure waters particularly in warm weather.

For these reasons, lactose-broth has in recent years been generally substituted for dextrose broth in the presumptive test. The 1923 edition of Standard Methods recommends that this test should be made in 0.5 per cent lactose broth with 48 hours incubation at 37° C. If more than 10 per cent of the volume of the closed arm (or inverted vial) is occupied by gas after 24 hours the presumptive test is regarded as positive. If no gas appears after 48 hours the test is negative. Any other result is regarded as a doubtful one which must be confirmed.

It will be noted that in this standard medium the amount of lactose is reduced to one half of one per cent. Burling and Levine (1918) have shown that in either glucose or lactose broth it is best to limit the concentration of carbohydrate to 0.5 per cent since in a higher concentration the colon group organisms tend to die off more rapidly than the aerogenes types and are therefore likely to be lost.

Hasseltine (1917) reports results which indicate that the proportion of positive gas tests which later fail of confirmation may be substantially reduced by sterilizing the lactose separately in aqueous solution and, after mixing it with the previously sterilized broth, heating the mixture in the Arnold for 30 minutes.

It is important also to point out that the present standard procedure ignores the volume of gas produced, so long as it exceeds 10 per cent. In the earlier days of the colon test a great deal of stress was laid upon the exact amount of gas, 25 to 70 per cent of the capacity of the closed arm being considered diagnostic of *Bact. coli*. The unreliable character of quantitative gas results has, however, been demonstrated by Fuller and Ferguson (1905), Longley and Baton (1907) and others. The many factors which influence the volume of gas formed in the fermentation tube have been made clear by Browne and Ackman (1917). They find that the amount of gas varies not only with the temperature, the time of incubation and the initial reaction of the culture medium, but also with the particular medium and the length of the inverted vial used for collecting the gas. Even when all conditions are apparently constant, wide variations occur between individual tubes.

The prompt formation of at least 10 per cent of gas seems, on the other hand, to be a point of very real importance. Levine (1920) finds that presumptive tests in which 10 per cent or more of gas appears in 24 hours generally are confirmed (98 per cent): if less than 10 per cent gas appears in 24 hours, 91 per cent are confirmed; if no gas appears in 24 hours but over 10 per cent appears in 48 hours, 73 per cent are confirmed; if there is less than 10 per cent gas after 48 hours only 45 per cent are confirmed. These figures are for untreated supplies, chlorinated supplies (as might be expected) showing much lower proportions of tests confirmed.

General Significance of the Presumptive Test. Even with the use of the lactose broth test, as defined above, a substantial proportion of positive presumptive tests may be obtained which are not due to organisms of the colon group. The commonest forms which give rise to such spurious presumptive tests are the anaerobic spore formers of the *Cl. welchii* type. Like the colon bacillus itself these bacteria are characteristic intestinal organisms and their use as possible indicators of pollution will be discussed

in a succeeding chapter. Being spore-formers, however, it is clear that they may survive for a long time in water and that their significance must therefore be very different from that of *Bacterium coli*. The proportion of presumptive tests associated with the presence of *Bact. welchii* rather than *Bact. coli* will therefore vary widely with different waters and will, in general, be highest where fresh pollution is least. Frost (1916) found that in polluted river water only 1 or 2 per cent of the positive presumptive tests were due to anaerobes but that in treated waters the proportion might rise to 13 or 14 per cent. Cumming (1916) reports the exceedingly interesting results presented in the table herewith which indicate the progressively decreasing percentage of gas tests confirmed as one proceeds down the river and self-purification takes place.

Station	No. of Samples	Average Bact. coli	Average Fermenting orgs. not Bact. coli	Percent non-coli to coli	Percent fermenting orgs. confirm'd as Bac. coli
Giesboro Point	770	295	24.1	8.1	92.5
Fort Foote	789	254	9.7	3.8	96.4
Fort Washington	778	123	6.2	4.8	95.2
Mt. Vernon to Whitestone Point	851	102	8.5	8.3	92.2
Indian Head	241	123	15.7	12.8	88.6
Possum Point	212	66.2	13.8	20.8	82.4
Maryland Point	681	1.44	.75	52.1	68.9
Pope's Creek	740	.19	.33	174.0	36.6
Lower Cedar Point	476	.14	.16	114.0	46.7
All below Lower Cedar Point	2261	.052	.057	110.0	47.7

Levine (1921) reports on a series of water analyses made on army service in France with the results indicated on p. 69, which show clearly the very slight value of the presumptive test with chlorinated waters.

On examination of the St. John River by the International Joint Commission on Boundary Waters it was found that anaerobic spore-formers were for some reason so common that of 444 positive presumptive tests 305 or 69 per cent were due to organisms of this type.

A second, though much less common, cause of spurious presumptive tests is the presence of a spore-bearing aerobic form, allied in many respects to *Bact. subtilis* but capable of forming

gas in lactose and other carbohydrate media. These organisms were first described by Meyer (1918) as isolated on eight different occasions from the tap water of Newport, Ky. They were later reported by Ewing (1919) from the city water supply of Baltimore, by Perry and Monfort (1921) and by Hinman and Levine (1922) from chlorinated surface waters in Iowa.

TABLE SHOWING CORRELATION OF RATE OF GAS PRODUCTION WITH CONFIRMATION OF THE PRESUMPTIVE TEST FOR COLON GROUP IN LACTOSE BROTH

Rate of Gas Production	Untreated supplies		Chlorinated supplies	
	Number of tubes showing gas	Percentage of gas tubes confirmed	Number of tubes showing gas	Percentage of gas tubes confirmed
Rapid (10 per cent or more in 24 hours)	684	97.7	57	44.0
Moderate (less than 10 per cent in 24 hrs.)	193	91.2	15	20.0
Slow (no gas in 24 hours; 10 per cent or more in 48 hours)	276	73.2	156	6.4
Very slow (no gas in 24 hours; less than 10 per cent in 48 hours)	33	45.5	26	0.0

Finally, it has been shown by Sears and Putnam (1923) that a positive presumptive test in the absence of colon group organisms may also be due to the symbiotic action of two different organisms neither of which alone can form gas from the original carbohydrate present. With lactose, for example, these investigators observed vigorous gas production in presence of *Streptococcus fecalis* and *Bact. paratyphi B.* In general this phenomenon was observed with pairs of organisms including one capable of forming acid from the primary carbohydrate, and another capable of forming gas from other substances. Media in which the first (acid-forming) organism has been grown alone, and which are then sterilized and inoculated with the second organism, fail to exhibit this phenomenon so that the authors conclude the symbiotic action depends on simultaneous utilization of certain intermediate products of metabolism.

Relative Value of Various Enrichment Media. Whether the incubation of a water sample in broth culture is used as a presumptive test or merely as a preliminary to the isolation and identification of the organisms concerned it is important to use a medium that shall, so far as possible, prove favorable to the *Bact. coli* group and unfavorable to all other organisms. We have seen that in this respect lactose broth is superior to dextrose broth, which it has now superseded. It is by no means certain, however, that some other media cannot be found which is better even than lactose broth, and it is desirable to consider in some detail the progress which has been made along this line.

In the past a great variety of enrichment media have been studied from this viewpoint among which may be mentioned the following: phenol broth (Irons, 1901; Reynolds, 1902): the Eijkman test, involving incubation at 46° C. (Eijkman, 1904; Christian, 1905; Neumann, 1906; Thomann, 1907; Nowack, 1907; Hilgermann, 1909; Konrich, 1910; Fromme, 1910): media containing neutral red (Rothberger, 1898; Makgill, 1901; Savage, 1901; Irons, 1902; Gage and Phelps, 1903; Stokes, 1904; Braun, 1906): aesculin media (Harrison and van der Leck, 1909; Hale and Melia, 1911): and liver broth (Jackson and Muer, 1911). None of these media has achieved any general popularity among American bacteriologists; but a great deal of attention has been devoted to the use of media containing bile salts, a procedure the development of which we owe principally to Jackson (1906).

MacConkey (1900) long ago suggested the use of media containing bile salts (sodium taurocholate) for the differentiation of *Bact. coli* and *Bact. typhosum*, and bile-salts media have been used by various English observers (MacConkey, 1901; MacConkey and Hill, 1901) for the isolation of sewage bacteria. Jackson studied the action of various bile media and showed their selective inhibitory action in the striking table quoted on page 71.

Jackson suggested the use of fresh ox bile containing 1 per cent of lactose as a presumptive test instead of dextrose broth. In particular he hoped that this medium would be free to a great degree from the negative results due to overgrowths in polluted waters. He reported 275 examinations of badly contaminated waters, in which 65 per cent of the samples failed to give the dextrose-presumptive test, and only 10 per cent failed to show gas in lactose bile. In a later communication, Jackson (1907)

reports that in the examination of 5000 samples of water at the Mt. Prospect Laboratory, the bile medium has proved uniformly satisfactory. He recommends incubation for 72 hours, results being commonly obtained, however, after 48 hours; and he considers any tube showing 25 per cent gas as positive.

SELECTIVE ACTION OF BILE SALTS
(Jackson, 1906)

	Bacteria per c.c.			
	Uncontaminated Well	Contaminated Pond	Suspension of Fæces	Suspension of Fæces
Gelatin, 20°	920	2700	350,000	900,000
Agar, 37°	25	170	450,000	900,000
Bile agar,* 37°	14	43	300,000	900,000
Lactose bile agar,*37°	0	25	250,000	675,000
Lactose bile agar,*37°	0	17	250,000	600,000
Bile agar, 37°	0	16	60,000	900,000

* Bile diluted, 1 : 1.

Like other enrichment methods which eliminate competing forms it is no doubt true that the lactose bile test cuts out some colon bacilli. As a presumptive method, however, it is far superior to dextrose broth, giving a higher proportion of positive tests with polluted waters and a lower proportion of erroneous positive tests with waters of good quality. In an examination of 176 surface waters in eastern Massachusetts, carried out under our direction, *Bact. coli* was isolated 70 times. The dextrose-broth test was positive 120 times, an error of 70 per cent; while the bile test, alone, was positive 78 times, an error of only 11 per cent. The tabulated results of these examinations indicate fairly the merits of the bile medium for preliminary enrichment and as a presumptive test.

PRELIMINARY AND COMPLETE RESULTS OF DEXTROSE BROTH AND BILE TESTS. 176 SURFACE-WATERS

	Preliminary Positive Results (Gas Formation)	Final Positive Results (Bact. Coli)
Dextrose broth	120	70
Lactose bile	78	64

Of course it must be remembered that the advantages of lactose-bile over dextrose broth are partly due to the inhibiting effect of the bile salts and partly to the use of lactose instead of dextrose which cuts out the dextrose-positive lactose-negative group to which allusion has been made earlier in the chapter. The relative importance of these two factors, lactose and bile, is well brought out in a study by Stokes and Stoner (1909). These authors have compared a considerable series of preliminary enrichment tests followed by final isolation in dextrose broth, lactose broth and lactose bile. Of 567 colonies from positive dextrose broth tubes only 52 per cent were colon bacilli; of 3752 colonies from positive lactose broth and lactose bile tubes, 88 per cent of the lactose broth colonies and 95 per cent of the lactose bile colonies were *Bact. coli.*

With sewages and heavily polluted waters in particular the lactose-bile medium has proved of the greatest value. When a large proportion of sewage is present the colon bacilli are fresh from the intestine and apparently able to resist the antiseptic salts. On the other hand, the high numbers of other bacteria present make the danger of overgrowths particularly great. With waters of fair quality, such as those with which we ordinarily deal in sanitary water analysis, lactose bile inhibits not only the overgrowing forms but the weaker representatives of the *Bact. coli* group itself, and the net effect is to diminish positive results.

Hale and Melia (1910) inoculated unsterilized water (shown to contain no gas formers) with a pure culture of *Bact. coli* and stored it for different periods and under different conditions, testing at intervals by various presumptive tests. The colon bacilli lived for 8–10 days at 37°, for 38–75 days at 20°, and 77–84 days at 8° C. Comparison of presumptive tests with plate counts on litmus-lactose agar showed that a gas test in dextrose broth corresponded to an average of 4 bacteria and a positive test in lactose bile to 39 bacteria. In general the dextrose broth showed gas in one dilution higher than the lactose bile; and the difference increased with the attenuation due to prolonged sojourn in water.

The conclusion that the colon bacteria inhibited by bile necessarily represent forms of remote intestinal origin is contested by Jordan (1913) and Cumming (1916). The latter investigator

found that, with sewage, lactose bile yielded only 25 per cent as many positive tests as lactose broth, while with river water the ratio rose to 50–70 per cent.

A considerable part of the confusion in regard to the value of bile enrichment media is no doubt due to the varying amounts of bile used by various observers, as has been pointed out by Levine (1922). Jordan, Obst and Cumming used original whole bile or 10 per cent dried bile while Hale used 5 per cent dried bile. Levine himself finds that 1–2 per cent dried bile inhibits most spore formers, both aerobic and anaerobic, while accelerating the growth of colon-group organisms; but higher concentrations prove distinctly inhibitory to the colon group itself. Salter (1919) finds that bile salts added to a one-half-per cent peptone medium in concentrations of .3–.5 per cent exert a stimulating action on the growth of *Bact. coli*, while at concentrations above .5 per cent these salts become markedly inhibitive. Schoenholz and Meyer (1921) throw a very interesting light on this problem by demonstrating that the influence of bile salts upon the typhoid bacillus varies markedly at different hydrogen ion concentrations. At a P_H value of 7.0 even 1 per cent of bile increases growth while at P_H 8.0, 0.5 per cent is inhibitive.

Ritter (1919) reports a series of 1899 samples examined with parallel enrichment in lactose broth and lactose bile and finds that when either bile or broth is negative *Bact. coli* is generally absent but that when both are positive 75 per cent of the samples give confirmatory tests for *Bact. coli*. When both media show gas in 24 hours confirmatory tests are positive in 98 per cent of the samples; the author believes that when this occurs confirmatory tests are unnecessary. Young, in the work of the Kansas State Water Survey has used an even more complicated procedure involving the simultaneous inoculation of lactose bile, lactose broth and dextrose broth.

Levine (1921) sums up the arguments, pro and con, in regard to lactose bile as follows:

"1. Lactose bile is a more reliable presumptive test but a greater proportion of the colon group may be detected by preliminary enrichment in lactose broth.

2. If the proper concentration of bile salts could be determined the bile medium would probably be preferable. For the present,

considering the difficulty of obtaining bile of constant composition or the chemically pure salts, and in view of our insufficient knowledge as to the optimum concentration of bile salts, it seems best to employ lactose broth as a more uniform medium may thus be obtained in different laboratories. It is very probable that if a standardized evaporated bile were available a concentration of 1 to 2 per cent in lactose peptone water would be superior to lactose broth."

It is probably in large measure for the reasons stated that the 1923 Standard Methods Report makes no mention of bile and recommends only lactose broth for use as a preliminary enrichment medium.

The Use of Inhibitive Dyes in the Isolation of the Colon Bacillus. A new and highly promising suggestion for the improvement of the presumptive test has been made by Hall and Ellefson (1918) who show (following the remarkable work of Churchman on the bacteriostatic action of dyes) that the addition of gentian violet to lactose broth eliminates a large proportion of the anaerobic gas-formers. The same authors (1919) later made a careful study of the inhibitive effect of gentian violet upon *Bact. coli* and other gas formers and found that increasing proportions of gentian violet in enrichment media tend to yield a larger and larger proportion of confirmation of presumptive tests but, as might be expected, also tend to diminish the absolute number of isolations by inhibiting the weaker strains of *Bact. coli*. A concentration of about 1 part gentian violet in 9000 parts of lactose broth gives practically 100 per cent presumptive tests confirmed but on the other hand the total proportion of samples yielding *Bact. coli* begins to fall off at concentrations of gentian violet above 1 part in 100,000. Gentian violet was found to be much less inhibitive for *Bact. coli* in glucose broth than in lactose broth.

Wagner and Monfort (1921) suggest the use of an enrichment medium containing no beef extract but 2 per cent peptone, 0.2 per cent lactose and 0.001 per cent gentian violet. The elimination of the beef extract is recommended in the interest of uniformity; the increased amount of peptone helps to make up for this omission; the reduction in sugar is held to be advantageous; and the authors recommend pasteurization instead of sterilization in the autoclave, relying on the gentian violet to inhibit spore formers.

Bronfenbrenner, Schlesinger and Soletsky (1920) find that rosolic acid is an excellent differential antiseptic for checking the development of Gram-positive organisms while permitting almost all Gram-negative organisms to grow readily. Even *Bact. dysenteriae* which is inhibited by brilliant green and crystal violet is resistant to rosolic acid. Finally Muer and Harris (1920) report that brilliant green in a dilution of 1 part in 30,000 will check the development of *Cl. welchii* in a bile medium while *Bact. coli* grows readily in the presence of 1 part in 350. He suggests a medium containing 5 per cent dried oxgall, 1 per cent peptone, 1 per cent lactose and .01 per cent brilliant green for the isolation of *Bact. coli* from water.

Winslow and Dolloff (1922) in a comparative study of these several triphenylmethane dyes which have been recommended by various investigators determined the limiting concentration of each dye in lactose broth and in a lactose bile medium. The latter contained 5 per cent of sodium cholate and was distinctly more favorable to the development of colon group organisms than lactose broth. All of the three dyes tested, rosolic acid, gentian violet and brilliant green, had about the same toxicity for *Bact. coli* in lactose bile, one part in 1000 proving inhibitive. Rosolic acid behaved in exactly the same way in lactose broth. Gentian violet on the other hand was from 5 to 50 times as toxic in lactose broth as in the bile medium, and brilliant green from 200 to 1000 times as toxic. *Bact. aerogenes* for instance was inhibited by one part of brilliant green in 100,000 parts of lactose broth and one *Bact. coli* strain by one part in a million.

These results are highly suggestive; and it seems to the authors very probable that further investigations along the lines here indicated may lead to the development of a presumptive test which shall be of substantial value.

Purple Agar. Another medium which seems to offer many advantages for the detection of organisms of the colon group is Brom-cresol-purple agar. Unpublished results obtained by one of us (S. C. P.) has indicated that these organisms produce a color change which may be easily observed within twenty-four hours and which seems to be more reliable than either lactose litmus agar or Endo. Further work is necessary, but it appears to be a distinct addition to the available media for water work.

Pure Cultures from the Enrichment Tube. In case one does not rely upon a "presumptive" test alone but desires to study the organisms present in detail, the isolation upon a solid medium must follow the enrichment process. Since the enrichment tube was inoculated with a known amount of water all further work is purely qualitative, and it is only necessary to obtain such a number of colonies upon the plate that the isolation of a pure culture shall be easy. With practice it is possible to effect a proper seeding by barely touching the tip of a straight needle to the broth in the fermentation tube and transferring this directly to the agar. The touch must be a very light one, however, or the colonies on the plate will be too numerous for proper isolation.

The first medium which gained general acceptance in this country for the isolation of the colon bacillus was litmus-lactose-agar, which has already been discussed in another connection in Chapter IV; but this medium has now been superseded by Endo agar or eosin-methylene-blue agar. When used to isolate *Bact. coli* the plates should be incubated for from 12 to 24 hours at 37° C., at the end of which time, if organisms of the colon group are present, typical red colonies will be visible.

If no red colonies appear on the plate after a positive result in dextrose broth one of four things has occurred: There may be an organism present which forms gas in dextrose but no acid in lactose; there may be present forms which individually fail to attack lactose but growing together, symbiotically, produce gas in dextrose; *Cl. welchii* or some other form which will not grow on aerobic plates may have produced the gas; or an organism originally present and capable of fermenting both sugars may have been overgrown and lost in the enrichment tube. If plates are made on the first appearance of gas the likelihood of the latter possibility will be reduced to a minimum. In general, therefore, the absence of red colonies on the agar plate may be considered a negative result. If red colonies are present they must be sub-cultured and examined further.

Meyer (1917) suggested the use of 3 per cent agar instead of standard agar in streaking from enrichment tubes in order to avoid the overgrowth of colon bacilli by spreaders. This stiff agar in his tests gave results nearly but not quite, as good as those obtained with Endo medium.

About ten years ago many bacteriologists began to use Endo medium instead of litmus-lactose-agar for the isolation of the colon bacillus. Various modifications were made in the original Endo formula, as a result of suggestions by Hasseltine (1917) Levine (1918) and others, and the formula given in 1923 Standard Methods in the Appendix was finally adopted.

Levine (1921) recommends "a modified and simplified Endo medium which requires no adjustment of reaction and which need not be filtered. The medium consists of 1 per cent Difco peptone, 0.3 per cent dipotassium phosphate, 0.5 per cent agar, and 1 per cent lactose. One-half c.c. of a saturated basic fuchsin, decolorized by 2.5 c.c. of a 10 per cent sodium sulphite, as recommended by the Hygienic Laboratory, is employed as an indicator for each 100 c.c. of the medium. Aside from the simplicity of preparation, an advantage claimed is that *Bact. coli* may be differentiated from *Bact. aerogenes*. The former possess a distinct metallic sheen, the colonies are flat and button-like, and about two or three m.m. in diameter; whereas the latter usually produces considerably larger colonies which are convex and a metallic sheen is rarely observed. The disadvantage of the medium is that diffusion of color, due to acid production, is very rapid. This may be reduced by increasing the content of agar but when that is done the differentiation between *Bact. coli* and *Bact. aerogenes* becomes less distinct. All of the Endo mediums above have the disadvantage of instability. Exposure to light or air induces a deep red coloration which interferes seriously with the detection of acid formers thus making it necessary to prepare the medium fresh and at frequent intervals."

Kahn (1918[a], 1918[b]) suggests that confirmatory tests in Endo medium should be made with slants instead of plates and that inoculation should be made by both stab and slant. Colon group organisms produce more typical colonies on the slant than on plates and form gas in the stab, while anaerobes neither grow on the surface nor form gas in the stab. There is of course a very obvious convenience in using slants instead of plates; and Kahn (1919) believes that he can generally differentiate between colon and aerogenes types on this medium, the former producing typical metallic colonies, the latter pink non-metallic colonies.

Levine (1918) has suggested the use of a modified eosin-methylene-blue agar (first used by Holt, Harris, and Teague) for the

isolation of the colon bacillus. On this medium he finds that the colonies of *Bact. coli* and *Bact. aerogenes* can be easily differentiated. " The medium is prepared in the following manner:

Distilled water	1000 c.c.
Peptone (Difco)	10 gm.
Dipotassium phosphate	2 gm.
Agar	15 gm.

Boil ingredients until dissolved and make up any loss due to evaporation. Place measured quantities in flasks and sterilize at 15 pounds for 15 minutes.

Just prior to use, add to each 100 c.c. of the melted agar, prepared as above, the following constituents:

Sterile (20%) lactose solution	(1 gm. lactose)	5 c.c.
Aqueous (2.0%) eosin (yellowish) solution		2 c.c.
Aqueous (0.5%) methylene blue solution		2 c.c.

DIFFERENTIATION OF *BACT. COLI* AND *BACT. AEROGENES* ON EOSIN–METHYLENE BLUE AGAR

	Bact. coli	Bact. aerogenes
Size	Well isolated colonies are 2–3 m.m. in diameter.	Well isolated colonies are larger than coli; usually 4–6 m.m. in diameter or more
Confluence	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance by transmitted light	Dark almost black centers which extend more than $\frac{3}{4}$ across the diameter of colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as *Bact. coli* and smaller in proportion to the rest of the colony. Striated internal structure, often striated in young colonies.
Appearance by reflected light	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than *Bact. coli*. Metallic sheen not observed except occasionally in depressed center when such is present.

Pour medium into petri dishes, allow it to harden in incubator and inoculate in the ordinary way. Smearing the surface with a glass rod seems preferable to the streaking method sometimes employed.

There is no adjustment of reaction and filtration of medium is not necessary.

Test tubes may be substituted for petri dishes if desired. The value of such a change is (1) the reduction of expense, as only 3 or 4 c.c. of medium is needed for every test tube while about 15 c.c. is usually employed with petri dish, and (2) test tubes may be stored for long periods whereas the medium in petri dishes would have to be prepared at intervals of a week or less."

The differences between typical *Bact. coli* and typical *Bact. aerogenes* colonies on this medium are indicated in the table, p. 78. Levine (1921) reports that examinations made in the Army laboratories in France confirm the conclusion that 94 per cent of the colonies picked out as *Bact. coli* and 85 per cent of those picked as *Bact. aerogenes* on eosin-methylene-blue-agar prove to be correctly identified. He also gives a description of several varieties of *Bact. coli* and *Bact. aerogenes* colonies which may be distinguished on this medium.

Comparative Work with Endo and Eosin-methylene-blue Agars. During the past year an extended bacteriological study of some of the public water supplies of New Hampshire has been carried out by Mr. C. L. Pool, sanitary engineer of the State Department of Health, the results of which have not been published. Most of the water supplies studied were natural waters used without filtration, disinfection or other treatment. In nearly half the water supplies storage for a short period is employed, while in the remainder the supplies are from streams used without storage. In this study the water was first subjected to a presumptive test with lactose broth and tubes yielding positive results were then further examined by inoculation upon Endo and eosin-methylene-blue agars (Levine's formula) for confirmation and to determine the type of organism present.

Both summer and winter conditions have been studied, although as might be expected, the number of samples examined was much greater in the former than the latter period. It may further be stated that the summer test covered a rainy period when the ground was saturated and run-off correspondingly high. The winter test was made when frozen conditions prevailed.

	Summer Period	Winter Period
Total samples examined (showing gas in one or more of the lactose broth tubes)	97	15
No. of tubes from above samples examined by eosin-methylene blue	198	24
No. of tubes examined on Endo	193	19
No. of tubes examined on both media simultaneously	192	19
on Endo alone	1	0
on eosin-methylene-blue alone	6	5

The confirmatory tests made with eosin-methylene-blue agar gave the results:

	Summer Period	Winter Period
Plates showed both *Bact. coli* and *Bact. aerogenes*	37	1
Plates showed *Bact. coli* alone	66	14
Plates showed *Bact. aerogenes* alone	56	2
Plates showed neither organism	39	7
	198	24
Percentage of + tubes showing *Bact. coli*	52.5	83.3
Percentage of + tubes showing *Bact. aerogenes*	47.5	16.7

Of the 192 summer tubes examined for " colon bacilli " by the two methods, only 4 failed to check, that is, indication of a positive result on Endo was accompanied by indication of either *Bact. aerogenes* or *Bact. coli* on eosin-methylene-blue, or vice versa, except in the four cases. Of the 19 winter tubes, none failed to check.

Comparing stored and unstored waters in a similar way for the summer period, Pool found the following:

	Stored	Unstored
Samples containing *Bact. aerogenes* alone	11, or 37%	8, or 23%
Samples containing *Bact. coli* alone	7, or 23%	16, or 46%
Samples containing both organisms	12, or 40%	11, or 31%
Totals	30, or 100%	35, or 100%
Total samples showing *Bact. coli*	19, or 63%	27, or 77%
Total samples showing *Bact. aerogenes*	23, or 77%	19, or 54.5%

Standard Routine Test for the Colon Group of Bacteria. The foregoing pages have indicated that the question of the best technique for isolating the colon bacillus is still an open one. It is still quite possible that by the right combination of bile salts and inhibitive dyes a medium may be devised which will give us a single presumptive test which is quite accurate enough for ordinary routine use. We are still not certain whether lactose broth is the best possible medium for preliminary enrichment. It is distinctly an open question whether eosin-methylene-blue agar should, or should not, supersede Endo agar for isolation. It is most important, however, to ensure such a measure of uniformity that results obtained by different observers may be reasonably comparable. Such uniformity will be best attained at present by close adherence to the procedure formulated by the Committee on Standard Methods in its 1923 Report, with such modification as may be approved by that committee in the future.

The 1923 Report prescribes that inoculations shall be made into standard lactose broth and the culture incubated at 37° C. for 48 hours. As pointed out above, the formation of gas occupying more than 10 per cent of the closed arm in 24 hours constitutes a positive presumptive test, the failure to form any gas in 48 hours constitutes a negative test, and any other findings a doubtful test.

Partial confirmation of the presumptive test is obtained by inoculating Endo medium or eosin-methylene-blue agar. If typical colon-like red colonies appear in 18–24 hours at 37° the partially confirmed test may be considered positive. If no typical colonies appear after 24 hours the plate is incubated for 24 hours more.

The final completed test is made by inoculating from each of two typical colonies on the 24-hour Endo or eosin-methylene-blue-agar plate, or from each of two colonies most nearly like *Bact. coli* on the 48-hour plate (if no typical colonies have appeared after 24 hours), a lactose broth fermentation tube and an agar slant. The formation of gas after 48 hours in this last lactose broth tube and the presence of non-sporing bacilli on the agar streak constitute final confirmation.

The committee on Standard Methods provides that the presumptive test shall be considered sufficient when positive: " (*a*) as applied to all except the smallest gas-forming portion of each

sample in all examinations"; and "(*b*) as applied to the smallest gas-forming portion in the examination of sewage or of water showing relatively high pollution, such that its fitness for use as drinking water does not come into consideration. This applies to the routine examinations of raw water in connection with control of the operation of purification plants." The partially confirmed test will be considered sufficient: "(*a*) when applied to confirm a doubtful presumptive test in cases where the latter, if definitely positive, would have been sufficient" and "(*b*) in the routine examination of water-supplies where a sufficient number of prior examinations have established a satisfactory index of the accuracy and significance of this test in terms of the completed test." In all other cases the completed test must be used.

Before leaving this subject of the technique of the colon test we desire to call attention to two laboratory devices which promise to be of real value to laboratories where a great volume of work is carried on. To facilitate the making of duplicate fermentation tests, Wells (1918) has described an improved form of apparatus (modeled on one devised by McCrady) which is in the form of a galvanized iron box with open front and back, holding ten test tubes (each with an inverted Wasserman tube within). The test tubes are not plugged but are closed by the cover of the box so that all ten may be quickly inoculated at one time.

From the standpoint of the State Laboratory which receives samples by mail from considerable distances we may note that McCrady (1920) has introduced in the Province of Quebec a novel plan by which daily samples for the determination of *Bact. coli* are mailed to the provincial laboratory. A special mailing case holds four cork-stoppered glass tubes each containing 2 c.c. of a concentrated enrichment medium, to which 5 c.c. of water is added by the water works operator. Inverted vials are inserted when the samples reach the laboratory. This method has been used in the analysis of some 18,000 samples and has proved highly satisfactory.

Differentiation between Bact. coli and Bact. aerogenes. We have reviewed on pages 56 to 60 some of the earlier studies in regard to the different significance of the two sections of the colon group, the low-ratio, methyl-red-positive, Voges-Proskauer-negative *Bact. coli* and the high ratio, methyl-red-negative, Voges-

Proskauer-positive *Bact. aerogenes*. Certain later studies should be cited which bear out the assumption that the habitat of these forms is so distinct as to warrant placing a different interpretation upon their presence. Thus Greenfield (1916) reports that of 116 colon group organisms isolated from untreated natural surface waters in Kansas, 76 per cent were acid to methyl red. With 131 filtered or disinfected waters the ratio fell to 70 per cent; for 158 ground waters, to 65 per cent; 19 samples of natural ice, to 32 per cent. The differences are slight but in the direction to be expected if the *Bact. aerogenes* type is considered as less closely associated with fæcal pollution than *Bact. coli*. So Johnson and Levine (1917) report the aerogenes-cloacæ types as the predominant organisms of the colon group in soils in the neighborhood of Ames, Iowa, and find them more abundant in soils upon which crops are growing than in fallow soils receiving similar soil treatment. Rogers (1918) has suggested a further differentiation of *Bact. aerogenes* into a fæcal type fermenting adonitol and a non-fæcal type which rarely attacks this carbohydrate. He showed by bottle experiments that the non-fæcal *Bact. aerogenes* type has a distinctly greater viability in water than has *Bact. coli* and found confirmatory evidence of superior resistance by the distribution of the organisms in natural waters.

Winslow and Cohen (1918[a]), in a series of bottle experiments, confirm these conclusions of Rogers, finding that a water suspension of *Bact. coli* and *Bact. aerogenes* originally containing 54 per cent of the former type showed only 29 per cent of this type after 60 days storage. On the other hand, in a series of 255 strains isolated by the same authors from waters of various types the ratio of aerogenes to coli types was just as low (24 per cent) in strains from waters of unpolluted character as in those from polluted sources, while in stored waters the ratio was even lower (Winslow and Cohen, 1918[b]).

Stokes (1919) reports less than 2 per cent of colon-like organisms from fæces and urine to be of the high ratio type while cereals showed 28 per cent of this type, milk and oysters, 42–44 per cent, water, 56 per cent, and various grains, 85 per cent.

Chen and Rettger (1920) made a particularly thorough study of this problem and found that of 467 strains of colon group organisms isolated from soils, 430 were of the *Bact. aerogenes* type, 17 of the *Bact. cloacae* type and only 20 proved to be *Bact. coli*

On the other hand, 173 fæcal organisms from man and animals were all of the *Bact. coli* type. These authors found that motility, indol production and fermentation of adonitol were of little practical value in differentiation but that the methyl red test, the Voges-Proskauer reaction and the uric acid test of Koser were of high differential value. Hinman (1920) gives analyses of a water in Iowa which showed that before treatment 41 per cent of the samples contained *Bact. coli*, 9 per cent *Bact. aerogenes*, and 8 per cent other gas formers in 1 c.c. portions. After filtration and chlorination practically all 1 c.c. samples were negative but in 10 c.c. portions 2 per cent showed *Bact. coli*, 1 per cent *Bact. aerogenes*, and 18 per cent other gas formers.

Wood (1920) in England has confirmed the work of the American investigators. Of 132 strains isolated by him from human and animal fæces, 95 per cent were methyl-red positive and Voges-Proskauer-negative (*Bact. coli* type); of 94 strains from milk 82 per cent were of the *Bact. coli* type; of 15 strains from cereals and grain, only 26 per cent. In a series of 231 water strains 67 per cent proved to be methyl-red positive. There were 66 samples in all containing *Bact. aerogenes* but no *Bact. coli* and no streptococci, and most of these samples were from deep wells and other sources which from the sanitary inspection were probably unpolluted.

Levine (1921) has prepared an exhaustive summary of the work of various observers upon this point. Of 2534 strains of colon group organisms isolated from human fæces by 13 different observers 5.9 per cent were of the *Bact. aerogenes* type, and of 1832 strains from animal fæces by 11 workers only 7.4 per cent were of the *Bact. aerogenes* type. On the other hand, of 1141 strains isolated from soil and grains by 8 observers 86.5 per cent proved to be *Bact. aerogenes*.

The 1923 Standard Methods Report, without committing itself as to the importance of a differentiation between the *Bact. coli* and *Bact. aerogenes* types, includes a procedure for their differentiation, a procedure which appears to us to be quite needlessly complicated. A single additional subculture in dextrose potassium phosphate broth for the methyl-red test would seem to be sufficient for all practical purposes; and we believe that such a test should be made in all cases where full knowledge of the sanitary quality of a water sample is desirable.

Examination of Waters Under Unusual Conditions. Since it is sometimes very desirable to make examination of water supplies for small army camps, pleasure camps, isolated dairy farms, etc., when the services of a laboratory are not available, a modification of the methods previously described may be found necessary.

" Hitchens (Hitchens, 1923) has described an arrangement for the examination of waters under conditions that render the usual laboratory procedure impossible, yet which will, in his opinion, meet all the requirements so far as the presumptive test for *Bact. coli* is concerned. This method is suggested especially for Army use. Specially constructed apparatus has been avoided, use being made of 15 c.c. (half-ounce) homeopathic vials, 20 × 70 m.m., and small tubes, 10 × 75 m.m. over all, closed at one end and inserted in a perforated cork (size 4, xxxx). The stopper is covered (just as is the standard bottle for the collection of water intended for bacteriological examination) with a muslin cap held in place by a copper wire. The bottle receives 1 c.c. of ten times concentrated standard lactose broth, and the whole is assembled and sterilized in the autoclave at 15 pounds pressure for fifteen minutes.

The formula for the broth used is:

Meat extract	3 gm.
Peptone	5 "
Lactose	5 "
Water	100 "

Each vial is to be marked or scratched to indicate the level to which it should be filled to contain just 10 c.c. of the water under test, that is, the mark should indicate a total volume of 11 cubic centimeters. The method suggested for using this device is as follows:

1. Each presumptive test of water made for the presence of members of the Bacterium coli group shall be made in five vials in order that the test may conform to " Standard Methods."

2. Each vial shall be filled in the ordinary manner as given in the Manual of the Medical Department, with only those changes (words italicized) necessitated by differences in the type of the collecting bottle. These vials are to be filled to the mark or scratch which indicates 10 c.c. (Par. 358, **Bacteriological Examinations,** M.M.D. 1916, Cor. to April 15, 1917, page 115.)

Samples of water for bacteriological examination should be collected in bottles furnished for the purpose. Each bottle is sterilized before

leaving the laboratory, and the *cork* stopper is protected by a piece of heavy sterilized muslin securely wired to the neck of the bottle. The stopper should not be removed until immediately before the bottle is filled.

In taking specimens from a faucet or pump (after emptying the supply pipes and connections conformably to par. 357) a small, gentle stream should be allowed to flow, the stopper taken out, the bottle grasped near the bottom, held in an upright position, and the stream permitted to flow into the bottle until it is filled to the **mark or scratch.** The stopper should then be replaced . . . . The stopper must be handled only by the . . . . cloth-covered top. The lip of the bottle must not be brought in contact with the faucet or spout, nor should the neck of the bottle or naked part of the stopper be permitted to come in contact with any object during the manipulation . . . . The stopper should not be laid down and the cloth should not be handled by the fingers except in the act of securing the wire about it. When well water is to be examined, the bottle should be filled directly from the bucket constantly in use for drawing the water, and from no other vessel.

3. The vial being filled to the mark, the stopper with its tube is placed in the bottle tightly and the contents are agitated to mix the water and the broth. If a few drops of water overflow, this is negligible. When inserting the stopper, let the open end of the tube rest against one side of the vial and hold this side down; then, tilting the bottle, let all the air flow out of the tube so that when the bottle is turned to the upright position the tube will be completely filled with water and contain not even a small bubble of air.

4. The tube being completely filled with water, place the vial in the upright position, loosen the stopper carefully, so that if there is gas formation within the vial it will have no difficulty in escaping. With the stopper placed loosely in the bottle the muslin cap will effectively prevent outside contamination.

5. If an incubator held at 37° C. is available, the five bottles may be placed in it and examined after twenty-four and forty-eight hours. If no incubator is available, room temperature will permit the growth of *Bacterium coli*, but vegetation will proceed more slowly at lower temperatures, the rate depending upon how near the temperature of the room approximates that of the body. With regard to the test, it is recommended by "Standard Methods" that the *Bacterium coli* "group" be considered as including all non-spore-forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media

The formation of 10% or more of gas in a standard lactose broth fermentation tube within twenty-four hours at 37° C. is *presumptive* evidence of the presence of members of the *Bact. coli* group, since the majority of the bacteria which give such a reaction belong to this group.

As *Bacterium coli*, if present, develops in the presumptive test vial, it will attack and decompose the lactose, the decomposition resulting in the formation of gas. The gas formed from growth within the inverted tube will collect therein and displace the broth. The amount of gas found in the

tube is recorded in terms of the proportion of broth displaced. For instance, if the tube is half full of gas, 50%, if one-fourth full, 25%. The following is quoted from "Standard Methods":

> Examine each tube at twenty-four and forty-eight hours, and record gas formation. The records should be such as to distinguish between:
>
> (*a*) Absence of gas formation.
>
> (*b*) Formation of gas occupying less than ten per cent (10%) of the closed arm (*i.e.*, inverted tube). (These tubes are inserted in the cork in such a way that at least 10% projects above the upper surface of the cork, thus facilitating the readings.)
>
> (*c*) Formation of gas occupying more than ten per cent (10%) of the closed arm.
>
> More detailed records of the amount of gas formed, though desirable for purposes of study, are not necessary for carrying out the standard tests prescribed.
>
> The formation with twenty-four hours of gas occupying more than ten per cent (10%) of the closed arm of fermentation tube constitutes a *positive presumptive test.*
>
> If no gas is formed in twenty-four hours, or if the gas formed is less than ten per cent (10%), the incubation shall be continued to forty-eight hours. The presence of gas in any amount in such a tube at forty-eight hours constitutes a *doubtful test,* which in all cases requires confirmation.
>
> The absence of gas formation after forty-eight hours' incubation constitutes a *negative test.* (An arbitrary limit of forty-eight hours' observation doubtless excludes from consideration occasional members of the *Bact. coli* group which form gas very slowly, but for the purposes of a standard test the exclusion of these occasional slow gas forming organisms is considered immaterial).

The "closed arm" referred to above is represented by the inverted tube of the "presumptive test vial."

Water may be considered to be potable if members of the *Bacterium coli* group are present in not more than one 10-c.c. quantity out of five, when planted in standard lactose broth and incubated at 37° C. for forty-eight hours. With an incubator available, the presumptive test may be completed within forty-eight hours, using five "presumptive test vials" and without any other laboratory equipment. If within forty-eight hours no gas appears in any of the five tubes, or if gas appears in only one of the five, the water is *potable* and no further laboratory study is necessary. If, however, gas is seen in two or more of the tubes after forty-eight hours' incubation at 37° C., the presumptive test is positive and further examination is necessary in order to determine the potability of the water.

When a 37° incubator is not available, it may be possible to improvise a satisfactory water bath, but if even this cannot be obtained the presumptive test vials may be allowed to stand at room temperature in the dark for a longer period. In any room, whose temperature is at a "habitable" point (70° F.±), not longer than three days will ever be required. If gas appears

in not more than one tube after three days in even a relatively cool room, the water may be declared *potable*. The lack of a thermometer is about the only condition which will render it impossible to improvise a water bath, however.

SUMMARY

To make the presumptive test for potability of water:

1. Take five "presumptive test vials" and add to each, by the method given above, approximately 10 c.c. of the water to be examined — *i.e.*, up to the mark or scratch on the vials.

2. Put the stopper back tightly, shake the vial, let the open end of the tube touch the side of the vial and, turning it on its side, completely fill the inner tube with the water and broth.

3. Turn the vial upright and loosen the stopper so that any gas formed during incubation may escape.

4. When all five of the vials have been filled, place them in an incubator or water bath at 37° C., or in a dark place approximating as nearly as possible the temperature of the body.

5. After twenty-four hours' incubation, examine the five vials and note whether or not there has been gas formation; if there has been, record the amount in each of the five vials. There will practically always be a tiny bubble of air in the top of the tube, due to separation of a part of the dissolved oxygen from the water during incubation; if there is not more, therefore, than a small bubble, this will be ignored.

6. The vials will be examined again after forty-eight hours' incubation, noting gas formation as at the twenty-fourth-hour examination.

7. If there is no gas formation — or less than 10% — in any of the five tubes, the water is *potable* and further examination of the sample is unnecessary.

8. If there is 10% of gas or more in *only one* of the five tubes, the water is *potable* and further bacteriological examination of the sample is unnecessary.

9. If there is 10% or more of gas in *two or more* of the tubes, further bacteriological examination is required to learn whether or not the water is potable, and meanwhile it should be chlorinated or iodized in order that there may be no doubt as to its safety for drinking.

10. Should an incubator at 37° C. not be available and should it be impossible to improvise one, holding the tubes at living-room temperature for not more than three days will be at least equivalent to forty-eight hours in the incubator."

In routine water examination the necessity for fresh or properly iced samples should be made clear and insistent. However, it sometimes happens in the practical examination of waters to de-

termine their sanitary quality that delays in transportation take place, or failure to ice the sample sufficiently occurs. While the figures for total numbers are without significance in such a case, owing to the probable increase of water bacteria and the possible changes in number of *Bact. coli*, the presumptive test for organisms of the colon type may still be made in many instances. Samples even two or three days old, showing positive presumptive tests, should be regarded as suspicious and demand made for a fresh suitably protected specimen for confirmatory analysis. On the other hand, a negative result in a sample not over three days old generally indicates the absence of *Bact. coli*, and the safety of the water, even though the total count at 20° C. may be very high.

Quantitative Statement of the Results of the Colon Test. The plate method yields results which may be expressed, with greater or less accuracy, in direct quantitative terms. The results of the colon test on the other hand give us merely the information that *Bact. coli* is absent from a certain amount of water or is present in a given proportion of samples of a certain volume. In 1907 Phelps suggested that the reciprocal of the highest dilution in which a positive result is obtained should be used as the expression of the number of colon bacilli present in the sample under examination. If a negative result were obtained in one dilution and a positive result in a higher dilution, the two results should be transposed. Thus, if positive results were obtained in 1.0 c.c and 0.1 c.c. with a negative result in 0.01 c.c. we should say there were present 10 colon bacilli per c.c. If the 1.0 c.c. and 0.01 c.c. tests were positive and the 0.1 c.c. negative we should reverse the two latter figures and again say there were 10 colon bacilli per c.c. This is the procedure which has been generally adopted since the 1917 Standard Methods report.

During the past few years there has been a somewhat exhaustive discussion of the applications of the mathematical theory of probabilities to this question, in which the work of McCrady (1915 and 1918), Wolman and Weaver (1917, and Wolman, 1920) Stein (1919 and 1921) and Wells (1918 and 1922) should be specially mentioned. It is unnecessary to discuss here any detailed analysis of the problem, but in general it appears to the authors that McCrady and Stein, whose work is in essential agreement, have presented the clearest and most convincing case, and that a quotation from the tables suggested by McCrady will

give what the average water bacteriologist needs for his practical work.

TABLE A

FERMENTING ORGANISMS IN 100 C.C. OF SAMPLE CORRESPONDING TO CERTAIN FERMENTATION-TUBE RESULTS

Using 2 tubes with each dilution

Positives with 10 c.c.	No.	Positives with		No.	Positives with			No.
		10 c.c.	1 c.c.		10 c.c.	1 c.c.	0.1 c.c.	
0	0	0	0	0	0	0	0	0
1	7	0	1	5	0	0	1	5
2	11+	0	2	10	0	1	0	5
		..	...	..	0	1	1	9
		1	0	6	0	..	..	..
		1	1	13	..	..	..	..
		1	2	20	1	0	0	6
		..	..	..	1	0	1	12
		2	0	25	1	1	0	13
		2	1	70	1	1	1	20
		2	2	110+	1	2	0	20
					1	2	1	30
					..	..	..	..
					2	0	0	25
					2	0	1	50
					2	1	0	60
					2	1	1	130
					2	1	2	200
					2	2	0	250
					2	2	1	700
					2	2	2	1100+

The essential point, which all these observers make, is that the Phelps' method of reciprocals does not state the true mathematical probabilities of the case. If, for example, a positive result is obtained in each of two 10 c.c. portions of water we may assume on sound mathematical grounds, not 10 but 11 colon bacilli to be present per 100 c.c. If one out of two 10 c.c. portions be positive, we can assume not 5 but 7 colon bacilli per 100 c.c.

The tables cited from McCrady's paper (1918) show the probable number of colon bacilli present, according to his formula, for all possible results in dilutions of 10 c.c., 1.0 c.c. and 0.1 c.c. when either two or three fermentation tubes are inoculated at each

dilution. The original paper gives similar statements for four and five tubes at each dilution and Stein (1919) has published an excellent graph from which similar computations may be made.

TABLE B

Using 3 tubes with each dilution

Positives with 10 c.c.	No.	Positives with		No.	Positives with			No.	Positives with			No.
		10 c.c.	1 c.c.		10 c.c.	1 c.c.	0.1 c.c.		10 c.c.	1 c.c.	0.1 c.c.	
0	0	0	0	0	0	0	0	0	2	2	1	30
1	4	0	1	3	0	0	1	3	2	2	2	35
2	11	0	2	6	0	1	0	3	2	2	3	40
3	14+	..	..	..	0	1	1	6	2	3	0	30
		1	0	4	0	2	0	6	2	3	1	35
		1	1	7	..	..	..	..	2	3	2	40
		1	2	12	1	0	0	4	..	..	..	...
		1	3	16	1	0	1	7	3	0	0	25
		..	..	..	1	0	2	11	3	0	1	40
		2	0	9	1	1	0	7	3	0	2	65
		2	1	15	1	1	1	11	3	1	0	45
		2	2	20	1	2	0	11	3	1	1	75
		2	3	30	1	2	1	15	3	1	2	115
		..	..	..	1	3	0	16	3	1	3	160
		3	0	25	..	..	..	..	3	2	0	95
		3	1	45	2	0	0	9	3	2	1	150
		3	2	110	2	0	1	14	3	2	2	200
		3	3	140+	2	0	2	20	3	2	3	300
					2	1	0	15	3	3	0	250
					2	1	1	20	3	3	1	450
					2	1	2	30	3	3	2	1100
					2	2	0	20	3	3	3	1400+

A second mathematical point, of considerable importance in connection with the statement of the results of the fermentation test, concerns the computation of a single figure to represent a series of examinations made on the same water over a considerable period of time. Stein and Wells differ radically in regard to this point, Stein suggesting an equation which represents the maximum number of *Bact. coli* to be expected in a series of 360 tests, Wells recommending the use of the geometric mean of the series of individual findings. All are agreed that the arithmetical average is so influenced by a few high results as to yield a distorted picture.

Wolman (1920), approaching this subject from the standpoint of the practical operator of a treatment plant, presents a graph of the results actually obtained in examining the water of the city of Baltimore showing the relation between the number of *Bact. coli* and the percentage of samples showing each colon content. The resulting curve is a straight line when plotted on logarithmic paper and we are inclined to believe that the analysis of curves of this character for each particular plant at which daily analyses are made will furnish the soundest basis for evaluating the condition of a water as judged by a long series of analyses.

CHAPTER VI

SIGNIFICANCE OF THE PRESENCE OF THE COLON GROUP IN WATER

Colon Bacilli in the Intestines of the Lower Animals. *Bacterium coli* is by no means confined to the human intestine. Dyar and Keith (Dyar and Keith, 1893) found it to be the prevailing intestinal form in the cat, dog, hog, and cow. About the same time, Fremlin (Fremlin, 1893) found colon bacilli in the fæces of dogs, mice, and rabbits, but not in those of rats, guinea pigs, and pigeons. Smith (Smith, 1895) recorded the presence of the organism, in almost pure cultures, in the intestines of dogs, cats, swine, and cattle; and he also found it in the organs of fowls and turkeys after death. Brotzu (Brotzu, 1895) reported *Bact. coli* and allied forms as very abundant in the intestine of the dog; and Belitzer (Belitzer, 1899) isolated typical colon bacilli from the intestinal contents of horses, cattle, swine, and goats. Moore and Wright (Moore and Wright, 1900) recorded the finding of the colon bacillus in the horse, cow, dog, sheep, and hen; and in a later report (Moore and Wright, 1902) they noted its occurrence in swine and in some, but not all, the specimens of rabbits examined. In frogs it was not found. Eyre (1904) isolated typical *Bact. coli* from the intestines of mice, rats, guinea pigs, rabbits, cats, dogs, sheep, goats, horses, cows, hens, ducks, pigeons, sparrows, divers, gulls, and fish of various sorts. Houston (1904) found *Bact. coli* abundant in the fæces of gulls, as might be expected from their feeding habits.

Where colon bacilli are isolated from presumably good water stored in an open reservoir, pollution introduced by birds must always be considered as a possibility, as in the case of the Los Angeles reservoir where Heinley (1916) reported an increase in colon bacilli due to flocks of ducks. Houston (1905) and other observers have found it impossible, even by the use of elaborate series of fermentation tests, to distinguish human *Bact. coli* from those found in animals. Savage (1906) compared colon-like

organisms isolated from the intestines of swine, cattle, horses, and sheep with those of human origin in respect to their action upon lactose, dulcite, mannite, raffinose, glycerine, maltose, galactose, lævulose, saccharose, starch and cellulose; but he failed to find any general correlations between habitat and biochemical powers.

Ferreira, Horta and Paredes (1908[b]) made an elaborate study of the distribution of colon bacilli in the lower animals. They isolated 81 lactose-fermenting bacilli from 38 species of mammals and 8 species of birds, including monkeys, bears, wolves, foxes, hyenas, lions, panthers, tapirs, a camel, deer, and ostriches from the Zoölogical Gardens. These cultures were studied by an elaborate series of tests and 93 per cent of them proved to be typical *Bact. coli*. Bettencourt and Borges (1908[b]) working in the same laboratory showed that there were no specific differences in agglutination with immune sera and in complement fixation between the colon bacilli of human and of animal origin. Konrich (1910) reports the examination of 170 samples of fæces from men, horses, swine, sheep, cows, goats, dogs, cats, guinea pigs, mice, rabbits, rats, earthworms, moles, fowls, swallows, sparrows, ducks, pigeons, geese, a jackdaw, a redstart, a blackbird, an adder, and a trout. Three out of 5 guinea pig samples, 4 out of 20 horse samples, 2 out of 3 mouse samples, 3 out of 8 rabbit samples, and 2 out of 8 earthworm samples, 14 in all, were negative; while all the rest showed *Bact. coli*.

In cold-blooded animals the occurrence of *Bact. coli* is less constant. Negative results in the frog and positive results in certain fishes, an adder and earthworms have just been quoted. Amyot (1902) failed to find the organism in the intestines of 23 fish representing 14 species. Johnson, on the other hand (Johnson, 1904), in the examination of the stomach and intestines of 67 fish caught in the polluted Illinois and Mississippi Rivers, isolated *Bact. coli* 47 times. He concluded from these results that the migration of fish from a contaminated stream or lake to an unpolluted one may explain the occasional finding of *Bact. coli* in small samples, or the more regular detection of it in large volumes of the water.

Bettencourt and Borges (1908[b]) isolated 29 cultures of colon-like microbes from the intestines of 17 types of fishes, reptiles and amphibia. Only 8 of the 29 formed gas in lactose broth and only 2 (from an eel and an adder) proved to be typical *Bact. coli*. It should be noted, however, that the samples of fæcal material

were plated directly on Endo medium instead of being subjected to the more sensitive process of preliminary enrichment.

Browne (1917) in a later study found that of 93 scup taken from generally unpolluted waters near Woods Hole, 27 contained *Bact. coli* organisms but not members of the *Cl. Welchii* group; 18, members of the *Cl. Welchii* but not of the *Bact. coli* group; 10, members of both groups.

Fromme (1910) reviews the work of many observers in regard to the presence of colon bacilli in the intestines of cold-blooded animals (particularly fish of various sorts and oysters) and concludes that while they are regularly found in warm-blooded animals they are found often, but not regularly, in cold-blooded animals. The lower the zoölogical type the rarer are the colon bacilli.

Colon Bacilli on Plants and Plant Products. The possibility was long ago suggested by various observers that the colon bacillus may live in a semi-parasitic fashion on plants as well as on animals. Of a series of 47 cultures of lactic-acid bacteria, examined by one of ourselves (Prescott, 1902[a]; Prescott, 1903, Prescott, 1906), 25 were found to give the reactions of the colon group. These organisms were isolated chiefly from cereals and products of milling, such as flour, bran, cornmeal, oats, barley, etc., while others were in technical use for producing the lactic fermentation. There is no evidence that any of these organisms were of intestinal origin, and yet they possessed all the characters of typical colon bacilli as then understood, even to the pathogenic action when inoculated into guinea pigs. In Germany, Papasotiriu (Papasotiriu, 1901) was meanwhile carrying on almost exactly similar investigations to Prescott's, with identical results.

Other testimony is somewhat conflicting with regard to the occurrence of *Bact. coli* on plants. Klein and Houston (1900) reported the finding of typical colon bacilli in only 3 out of 24 samples of wheat and oats obtained from a wholesale house; rice, flour, and oatmeal bought at two different retail shops gave *Bact. coli* in all three cereals in one case and on none in the other. Clark and Gage (1903) were unable to isolate *Bact. coli* from standing grains. Gordan (1904) could not find *Bact. coli* in .1 and .01 mg. samples of clean bran, but isolated it easily from that of poor quality. Winslow and Walker (1907) reported the examination of 178 samples of grain and 40 samples of grasses for

Bact. coli without success. On the other hand, Düggeli (1904) found *Bact. coli* among the bacteria occurring on the leaves of growing plants, although it was not one of the most abundant species. Barthel, too (Barthel, 1906), found *Bact. coli* widely distributed on plants from both cultivated and uncultivated regions. Bettencourt and Borges (1908[a]) examined 35 samples of vegetables and cereals purchased in open market and found 12 lactose fermenters, of which 6 proved to be *Bact. coli.*

Neumann (1910) has recently studied the distribution of colon bacilli on and in various food substances such as bread, milk, butter and fruit. From fresh fruits immediately after picking he never isolated them, but they were present in a certain proportion of all the foods which had been exposed to human contamination and the author concludes that wherever human hands have been, there will *Bact. coli* be found. Konrich (1910) in a similar series of investigations obtained positive results from 46 out of one hundred .1 to .5 gm. samples of cultivated plants while leaves of trees and grasses and herbs on waste places gave about 6 per cent positive results. Hay showed colon bacilli in 91 per cent of the 135 samples examined and grains in 55 per cent of 300 samples.

The work of Rogers and his associates, to which reference has been made in the preceding chapter, indicates that in the majority of instances the colon group organisms isolated from grains will be found on careful study to be of the *Bact. aerogenes* and not of the true *Bact. coli* type. Out of 288 strains isolated from grains and cereals four recent investigations (cited by Levine, 1921) 82 per cent were of the *Bact. aerogenes* type.

Colon Bacilli in Dust and Soil. Winslow and Kligler (1912) have shown that colon bacilli may be very abundant in the dust of city streets and houses, as might naturally be expected from the fact that such dust is largely made up of horse droppings. They examined 24 samples of street dust and 72 samples of house dust (all in New York City). All of the street dusts and 63 of the 72 house dusts contained colon bacilli in at least one of three duplicate .01 gram portions. In two street samples the numbers rose to 330,000 and 660,000 per gram respectively, while the largest indoor result was 60,000. The average for the indoor dusts was between 1000 and 2000 per gram and for the street dusts over 50,000 per gram. This dust was dust deposited on

surfaces and would only be carried up into the air by currents of some force. It is well known that colon bacilli are, as a matter of fact, rarely present in street or house air. Konrich (1910) exposed open Petri dishes of dextrose broth to the air of Jena streets for 24-hour periods, daily, for 3 months and found colon bacilli only 11 times. The colon bacilli in street dust may, however, perhaps account for the anomalous positive results sometimes obtained in reservoirs bordered by roadways.

Konrich (1910) has also made important contributions to the study of colon bacilli in the earth. Out of 547 samples of soil, 65% showed *Bact. coli* in portions of between .1 and .5 gm. The farther removed from cultivation a sample was, the less were the chances of positive results. He concludes that *Bact. coli* is widely distributed in the outer world. It is almost always found in soil from cultivated fields or from traveled places. The farther a source is removed from travel and from cultivation the more rarely is the colon bacillus found; but it is never altogether absent. On plants or parts of plants it is frequently found when they come from cultivated land; on plants from waste places it is rarely found. It seems probable that colon bacilli may be even more widely distributed in a thickly settled and intensively cultivated country like Germany than in the United States.

Here, as in the case of cereals and grains, recent work indicates that colon-like organisms found in soils are generally of the *Bact. aerogenes* type (88 per cent of a series of samples cited by Levine, 1921). Since the bulk of the results in the literature make no distinction between these two sections of the colon group, we shall ignore their relative significance and refer in the following discussion to the entire group of lactose-fermenting non-spore-forming bacilli.

The Number of Colon Bacilli, not their Mere Presence, as an Index of Pollution. From the data which have been cited, it seems clear that bacteria of the colon group are present in large numbers in human and animal fæces and also present though probably in smaller numbers, in soils and on certain plants. That they are not typical water organisms is clear from such investigations as those of Winslow and Cohen (1918) and Cohen (1922) which indicate that colon bacilli die off in water at a logarithmic rate in proportion to the time of storage. A few individuals may survive for very long periods as shown by Teissier and Convreur

(1919) who isolated *Bact. coli* from a bottle of water which had stood in the laboratory (protected against light) for a period of twenty years. The great majority of colon bacilli will however perish in natural waters in a few days or weeks.

When, therefore, we find a single colon bacillus in water (defining the colon bacillus in the usual broad sense) it may have come immediately from human fæces or it may have fallen into the reservoir on a leaf weeks before. The real question is whether colon bacilli from non-fæcal sources do, or do not, appear in unpolluted waters in large numbers. This is a question which can be settled only on the basis of practical experience in the examination of large numbers of representative waters from various sources. Such experience is at hand, in volume amply to demonstrate the significance of the colon bacillus when present in considerable numbers; but the interpretation of its significance must always be made in the light of the following considerations:

1. Bacteria corresponding in every way to *Bact. coli* (as ordinarily defined) are by no means confined to animal intestines, but are widely distributed elsewhere in nature.

2. The finding of a few colon bacilli in large samples of water, or its occasional discovery in small samples, does not necessarily have any special significance.

3. The detection of *Bact. coli* in a large proportion of small samples (1 c.c. or less) examined is imperatively required as an indication of *recent* sewage pollution.

4. The *number* of colon bacilli in water rather than their *presence* should be used as a criterion of recent sewage pollution.

With these qualifications the value of the colon test was never more firmly established than it is today. It seems clear that the colon bacillus finds in the intestine of the higher vertebrates an environment better suited to its growth and multiplication than any other which occurs in nature. Houston (1903[a]) records the number of *Bact. coli* per gram of normal human fæces as between 100,000,000 and 1,000,000,000. It is almost certain that the only way in which large numbers of these organisms gain access to natural waters is by pollution with the domestic, industrial, and agricultural wastes of human life. If pollution has been recent, colon bacilli will be found in comparative abundance. If pollution has been *remote* the number of colon bacilli will be small, since there is good evidence that the majority of intestinal bac-

teria die out in water. If derived from cereals or the intestines of wild animals, the number will be insignificant except perhaps where the water receives refuse from grist-mills, tanneries, dairies, or lactic-acid factories.

The first recognition of the necessity for a quantitative estimation of colon bacilli in water we owe to Dr. Smith, who in 1892 (Smith, 1893[a]) outlined a plan for a study to be made by the New York Board of Health on the Mohawk and Hudson Rivers. Burri (Burri, 1895) pointed out that the use of so large a sample as a liter for examination would lead to the condemnation of many good waters. Freudenreich (Freudenreich, 1895) at the same time indicated the necessity for taking into account the *number* of colon bacilli present. He recorded the isolation of the organisms from unpolluted wells, when as large a quantity of water as 100 c.c. was used, and concluded that it was entirely absent only from waters of great purity and present in large numbers only in cases of high pollution. This author also quoted Miquel as having found colon bacilli in almost every sample of drinking-water if only a sufficient portion were taken for analysis.

The practical results of the application of the colon test from this standpoint have proved of the highest value. As originally outlined by Dr. Smith, it consisted in the inoculation of a series of dextrose tubes with small portions of water, tenths or hundredths of the cubic centimeter. It was first used by Brown (Brown, 1893) in 1892 for the New York State Board of Health, and it showed from 22 to 92 fæcal bacteria per c.c. in the water of the Hudson River at the Albany intake, and from 3 to 49 at various points in the Mohawk River between Amsterdam and Schenectady. In some previous work at St. Louis, the colon bacilli in the Mississippi River were found to vary from 3 to 7 per c.c.

Hammerl (Hammerl, 1897) used the presence of *Bact. coli* as a criterion of self-purification in the river Mur. He considered, in spite of the position taken by Kruse, that when a water contained large numbers of colon bacilli, as well as an excess of bacteria in general, it might be considered to be contaminated by human or animal excrement. As, however, the organism would naturally be present in large quantities of such a water as that of the Mur, he used no enrichment process, but made plate cultures direct; In general, Hammerl failed to find colon bacilli in the river except immediately below the various towns situated upon it; at these

points of pollution he discovered a few colon colonies upon his plates, not more than 4 to 6 per c.c. of the water. He concluded that "the *Bacterium coli*, even when it is added to a stream in great numbers, under certain circumstances disappears very rapidly, so that it can no longer be detected in the examination of small portions of the water."

Very important work upon the distribution of *Bact. coli* has been that carried out in England by the bacteriologists of the local government board, Dr. Houston in particular. This investigator (Houston, 1898; Houston, 1899[a]; Houston, 1900[a]) made an elaborate series of examinations of soils from various sources to see whether the microbes considered to be characteristic of sewage could gain access to water from surface washings free from human contamination. In the three papers published on this subject the examination of 46 soils was recorded. In only 10 of the samples was *Bact. coli* found, and of these 10, 9 were obviously polluted, being derived from sewage fields, freshly manured land, or the mud-banks of sewage-polluted rivers. The author finally concluded that "as a matter of actual observation the *relative abundance* of *Bact. coli* in pure and impure substances is so amazingly different as to lead us to suspect that not only does *Bact. coli* not flourish in nature under ordinary conditions, but that it tends to even lose its vitality and die." Pakes (Pakes, 1900) stated on the strength of an examination of "about 300 different samples of water," no particulars being published, that water from a deep well should not contain *Bact. coli* at all, but that water from other sources need not be condemned unless the organism was found in 20 c.c. or less. When colon bacilli were found only in greater quantities than 100 c.c. the water might be considered as probably safe. Horrocks (Horrocks, 1910), after a general review of English practice, concluded that "when a water-supply has been *recently* polluted with sewage, even in a dilution of one in one hundred thousand, it is quite easy to isolate the *Bact. coli* from 1 c.c. of the water." "I would say that a water which contained *Bact. coli* so sparingly that 200 c.c. required to be tested in order to find it had probably been polluted with sewage, but the contamination was not of recent date." Chick (Chick, 1900) found 6100 colon bacilli per c.c. in the Manchester ship canal, 55–190 in the polluted River Severn, and numbers up to 65,000 per gram in roadside mud. On the other

hand, of 38 unpolluted streams and rivulets, 31 gave no *Bact. coli* and the other 7 gave 1 per c.c. or less. The Liverpool tap water, snow, rain, and hail showed no colon bacilli.

Colon Bacilli in Surface Waters. One of the first elaborate applications of the colon test was made by Jordan in the examination of the fate of the Chicago sewage in the Desplaines and Illinois Rivers. (Jordan, 1901.) The results were very significant. In fresh sewage a positive result was obtained about one-third of the time in one one-hundred-thousandth of a cubic centimeter and almost constantly in one ten-thousandth of a cubic centimeter. The Illinois and Michigan canal proved almost as bad, giving positive results on 7 days out of 28 in dilutions of one in a hundred thousand and on 28 days out of 32 in a dilution of one in ten thousand. At Morris, 27 miles below Lockport, where the canal enters the bed of the Desplaines River, and 9 miles below the entrance of the Kankakee, the principal diluting factor, the numbers were so reduced that positive results were obtained only on 11 days out of twenty in one thousandth of a cubic centimeter, on 20 days out of thirty in one hundredth of a cubic centimeter, and on 20 days out of 23 in one tenth of a cubic centimeter. At Averyville, 159 miles below Chicago, colon bacilli were isolated on only 4 days out of 27 in one tenth of a cubic centimeter, and on 13 days out of 31 in one cubic centimeter. A comparison with certain neighboring rivers showed this to be about the normal value for waters of similar character, as the following table extracted from Professor Jordan's paper will show:

NUMBER OF BACT. COLI PRESENT IN CERTAIN RIVER WATERS

(Jordan, 1901)

Source of Sample	0.1 c.c.		1 c.c.	
	No. Days Water Examined	No. Days Bact. Coli Found	No. Days Water Examined	No. Days Bact. Coli Found
Illinois River, Averyville	27	4	31	13
Mississippi River, Grafton	34	10	35	23
Fox River	22	2	23	6
Sangamon River	25	14	27	21
Missouri River	32	13	31	21

These results harmonize rather closely with those previously recorded by Brown and Fuller and indicate that in the larger rivers where the proportionate pollution is not extreme, colon bacilli may be isolated in about half the 1-c.c. samples examined. Such rivers are of course inadmissible as sources of water-supply, according to modern sanitary standards, unless subjected to purification of some sort.

Clark and Gage (1903) have published the results of certain studies of Massachusetts ponds which indicate clearly the coincidence of the distribution of *Bact. coli* in single centimeters of surface waters, with actual sanitary conditions. They show also the slight significance of the test for this organism in larger volumes of water. Almost every source gave positive tests in 100 c.c., while with 1-c.c. samples only those lakes appear suspicious which are, in fact, exposed to dangerous pollution.

DISTRIBUTION OF TOTAL BACTERIA AND BACT. COLI IN SURFACE-WATERS

(Clark and Gage, 1903)

Lake	Population of Watershed per Square Mile	Bacteria per c.c.	Bact. Coli Per Cent Positive Tests	
			1 c.c.	100 c.c.
1*	1400	612	13.3	33.0
2	356	319	3.5	17.2
3	116	103	0.0	0.0
4	90	170	0.0	14.0
5	62	87	0.0	9.0
6*	60	48	2.3	4.5
7*	50	66	4.6	21.0
8	47	133	0.0	9.0
9	42	131	0.0	6.7
10*	40	31	0.0	6.2
11	8	28	0.0	7.7
12	42	107	0.0	9.3

* Shores used for pleasure resorts.

Houston (1905) gives the following table, which may be taken as another fair example of the distribution of *Bact. coli* in small streams and lakes. Of the two lakes studied, Loch Ericht is free from the pollution of human or domesticated animals, while Loch Laggan receives some drainage from farm lands; both are

of large size. The brook and river samples were collected from adjacent streams.

DISTRIBUTION OF BACT. COLI IN SURFACE-WATERS

(HOUSTON, 1905)

Percentage of Samples showing Bact. Coli in each Dilution.

Dilution	+0.1 c.c.	+1.0 c.c. −0.1 c.c.	+10 c.c. −1 c.c.	+100 c.c. −10 c.c.	Not in 100 c.c.
Brooks and river	7.7	53.8	34.6	3.8	....
Loch Laggan	....	1.2	33.0	49.4	16.4
Loch Ericht	....	....	1.0	19.0	80.0

As an example of a heavily polluted stream, on the other hand, the table given below may be cited. It shows in a striking way the increase of *Bact. coli* in the Thames on its passage through London and its progressive purification below.

The river at the lower stations in this table was considerably diluted with sea-water, yet it showed clearly its large proportion of sewage. Normal sea-water, even in the neighborhood of the shore, shows *Bact. coli* only in large samples. Houston (1904), in another communication, reports the examination of 168 samples of sea-water near the English coast. None of the samples showed *Bact. coli* in 1 c.c.; 97 samples gave negative results in 10 c.c.; 45 in 100 c.c., and 4 had no *Bact. coli* even in 1000 c.c.

BACT. COLI IN THE RIVER THAMES AT VARIOUS POINTS

(HOUSTON, 1904[a])

Percentage of Positive Results

Place	−10 c.c.	+10 −1 c.c.	+1 − 0.1 c.c.	+0.1 −0.01 c.c.	+0.01 −0.001 c.c.	+0.001 −0.0001 c.c.	+0.0001 −0.00001 c.c.
Sunbury	....	....	....	70.6	23.5	5.9	.....
Hampton	....	....	11.8	64.7	17.7	5.9	.....
Barking	....	....	....	4.2	45.8	45.8	4.2
Crossness	....	....	....	11.1	27.7	50.0	11.1
Purfleet	....	....	3.0	9.1	33.3	39.1	15.1
Grays	....	....	2.8	22.2	41.7	33.3	
Mucking	....	....	30.8	57.7	11.5	.....	
Chapman	5.0	45.0	50.0	....	....		
Barrow Deep	12.0	36.0	40.0	12.0	....		

Gärtner (1910) has collected some interesting data in regard to the ratio between the number of colon bacilli and total bacteria in waters of different quality. The results from four different sets of experiments by Konrich at Jena, Houston at London, Noble in New York, and Hill at Giessen, may be combined as on the following page:

RATIO OF TOTAL BACTERIA TO COLON BACILLI IN WATERS OF DIFFERENT CLASSES

Coli titer — Smallest Portion of Water Showing Bact. Coli	Ratio of Plate Count to Bact. Coli					
	Jena	London		New York		Giessen
	20°	20°	37°	24°	37°	
100 c.c.	.....	60,500	11,800			
10 c.c.	695	15,100	1,810	1950	280	
1 c.c.	183	1,288	255	213	47	
0.1 c.c.	29.7	352	34	20	7	
0.01 c.c.	4.1	46	2.4	.....	.....	87
0.001 c.c.	.....	.....	.....	.....	.....	20
0.0001 c.c.	.....	.....	.....	.....	.....	4.9
0.00001 c.c.	.....	.....	.....	.....	.....	0.4

The rather regular decrease in the ratio of the total count to the *Bact. coli* count with an increase in the actual number of colon bacilli is very interesting.

Prof. Gärtner apparently holds that this fall in the ratio of the plate count to the " coli titer " indicates a fallacy in the method of the latter and in particular he emphasizes the absurdity of the lowest figures in the table which indicates that there were twice as many colon bacilli as bacteria of all sorts. It seems to us that the last phenomenon is quite as likely to be due to an error in the plate count as to a failure in the enrichment procedure. Unless dilutions are very carefully made plates inoculated with waters containing tens and hundreds of thousands of bacteria per c.c. are pretty likely to be so crowded that only a portion of the bacteria with which they are sown are able to develop. As to the diminishing ratio with increasing coli-content, it is exactly what might reasonably be expected. One-tenth to one-quarter of the bacteria in sewage may be colon bacilli, and, the greater the

amount of sewage present in water, the more nearly will this ratio be approached.

In the study of the Potomac River Cumming (1916) reports illuminating data in regard to the influence of self-purification upon the colon content of water. Just below the city *Bact. coli* ranged from 24 to 617 per c.c. (monthly averages), according to season; at Marshall Hall, 14 miles below, the averages ranged from 24 to 137; at Maryland Point, 42 miles below, from 0.04 to 6.9; and in the salt water stretch, 64–102 miles below, from 0.01 to 0.04 per c.c.

Colon Bacilli in Ground-waters. With ground-waters the story is the same. Even in sources of excellent quality we should expect to find, and we do sometimes find, colon bacilli in large volumes of water. Abba, Orlandi, and Rondelli (1899) showed by experiments with *Erythrobacillus prodigiosus* at Turin that when bacteria are present in great numbers on the surface of the ground, a few may penetrate for a considerable distance and ultimately reach the sources of ground-waters. The chance that disease germs could survive this process in a soil so impervious as to allow colon bacilli to appear only in large samples of water, is infinitesimal.

An interesting contribution to the bacteriology of ground-waters was made by the Massachusetts State Board of Health (Massachusetts State Board of Health, 1901) in connection with the examination of the spring-waters bottled for the sale in the State. Ninety-nine springs were included in this study, and in almost every instance 4 samples were examined, 2 taken directly from the spring by the engineers of the board and 2 from the bottles as delivered for sale to the public. In the water of one spring *Bact. coli* was found twice, once in a sample from the spring and once in the bottled sample. This spring was situated in woodland, but was unprotected from surface drainage, and the method of filling bottles subjected it to possible contamination. In 5 other cases *Bact. coli* was found once in the sample from the spring; all were subject to pollution from dwellings or cultivated fields, and 4 of the 5 were shown to be highly contaminated, chemically. In 7 other cases *Bact. coli* was found in the bottled samples alone; 3 of these sources were of high purity, but the bottling process furnished opportunity for contamination.

Clark and Gage (1903), in the examination of 170 samples of water from tubular and curb wells of good quality used as sources

of water-supply, found *Bact. coli* only 5 times, once in 1 c.c. and 4 times in 100 c.c.

Houston (1903[b]) makes an instructive comparison of some more or less polluted shallow wells at Chichester with deep groundwaters of high quality at Tunbridge Wells. The following table shows the value of the 1 cubic-centimeter sample in discriminating between good and bad waters.

DISTRIBUTION OF BACT. COLI IN GOOD AND BAD WELL WATERS
(HOUSTON, 1903[b])
Percentage of Positive Tests

Quantity of Water	Chichester Shallow Wells	Tunbridge Wells, Deep Wells
100 c.c.	90	25
10 c.c.	80	6
1 c.c.	45	0
0.1 c.c.	20	0

In a subsequent investigation, Houston (1905) examined still larger samples of water from the Tunbridge Wells for *Bact. coli:* 49 samples of 100 c.c. each showed no *Bact. coli*, and 27 liter samples showed *Bact. coli* only once. Kaiser (1905) reports an interesting correlation between total numbers and *Bact. coli* in a series of 38 well waters. Of 11 wells containing over 200 bacteria per c.c. 90 per cent showed colon-like organisms in liter samples. Of 12 wells containing from 50 to 200 bacteria per c.c. 67 per cent gave colon-like organisms; of 26 wells with less than 50 bacteria per c.c., only 27 per cent showed positive results.

Fromme (1910) brings out the relation between *Bact. coli* and total numbers in 120 samples of well waters near Hamburg in the table below.

RELATION BETWEEN TOTAL NUMBERS OF BACTERIA AND BACT. COLI
(FROMME, 1910)

Colony Count	Number of Samples	Per cent Positive Bact. coli Tests in 10 c.c.
Over 200	35	40.0
50–200	19	15.8
Under 50	66	3.0

Similar data obtained by one of us for some American sources have been cited in Chapter I. Even Konrich (1910), who is exceedingly sceptical as to the value of the colon test, has shown that an increase in the colon content of the Jena water supply (a groundwater) always followed a heavy rain which washed through some of the colon bacilli in the soil.

Colon Bacilli in Filtered Waters. One of the most important applications of the colon test is in the control of the operation of municipal water filters. It has been used for this purpose for 25 years or more at Lawrence, and Fuller laid stress upon its results in his classic experiments on water purification in the Ohio valley. At Cincinnati he records the presence of colon bacilli in 60 per cent of the 1-c.c. samples from the Ohio River, while the effluent from either slow sand or mechanical filters gave positive results only half the time in samples of 50 c.c. The results of the examinations carried out at Lawrence for 6 years are brought together in the table below from the Annual Reports of the Massachusetts State Board of Health.

BACT. COLI IN MERRIMAC RIVER AND LAWRENCE FILTER EFFLUENT

	Merrimac River, Per cent of 1 c.c. Samples Containing Bact. coli	Merrimac River, Number Bact. coli per c.c.	Filtered Water, Per cent of 1 c.c. Samples Containing Bact. coli
1900	99.7	87	18.1
1901	*	*	*
1902	99.0	73	4.0
1903	99.0	78	4.2
1904	100.0	73	8.0
1905	100.0	118	4.7

* Not given.

At Harrisburg, Pa., mechanical filtration combined with chlorin disinfection yielded the results tabulated on page 108.

In regard to the proportion of positive colon tests permissible in a filter effluent, Clark and Gage (Clark and Gage, 1900) reported some specially instructive observations made when certain of the underdrains of the Lawrence filter were relaid in the autumn of 1898. In doing this work the sand on some of the beds was seriously disturbed; and in December, after the work was

completed, *Bact. coli* was found in 1 c.c. of the filtered effluent in 72 per cent of the samples examined. In January and February the organisms were found in 54 per cent and 62 per cent of the samples, respectively, while in March the number fell to a value of 8 per cent. Corresponding to this excess of *Bact. coli* in the city water, there were 12 cases of typhoid fever in December, 59 cases in January, 12 in February, and 9 in March, all during the early part of the month. The authors conclude that " when filtering a river-water as polluted as that of the Merrimac, it is safe to assume that when *Bact. coli* is found only infrequently in 1 c.c. of the effluent, the typhoid germs, necessarily fewer in number and more easily removed by the filter, have been eliminated from the water."

BACT. COLI IN RAW AND TREATED WATER AT HARRISBURG, PA.

(HARRISBURG, 1913)

Year	Per cent Positive Tests in 1 c.c.	
	Raw Water	Treated Water
1906	71.9	2.7
1907	64.0	1.0
1908	65.7	1.1
1909	63.1	1.0
1910	55.4	0.2
1911	77.3	0.6
1912	46.9	0.8

The results of the daily tests carried out at municipal filter plants are frequently expressed in monthly or yearly averages, as in some of the cases quoted above. It must be remembered, however, that averages of this sort are accepted only by courtesy and with the implied assumption that conditions are approximately constant during the period averaged. When it is said that an acceptable effluent may show *Bact. coli* in 2 or 4 per cent of the samples tested the statement is true only for a series of samples collected and examined at the same time. If in a given month 3 per cent of the 1 c.c. samples tested show *Bact. coli*, the effluent may or may not be safe. If on each of 20 days 3 *Bact. coli* or thereabouts were present in 100 c.c. of the water it is probably a safe one. If

on 19 days no *Bact. coli* were present, and on the twentieth day 100 c.c. showed 60 *Bact. coli*, the average result would be the same, but the water on one day was of a dangerous character. With properly managed filter plants marked variations do not occur from day to day and average results are generally reliable. It is wholly misleading, however, to compare such results with the average examinations of an unfiltered surface water. With surface waters daily variations are the rule and a low monthly average of colon tests may include and cover up dangerous and significant high numbers at particular periods.

Altogether the evidence is quite conclusive that the absence of *Bact. coli* demonstrates the harmlessness of a water as far as bacteriology can prove it. That when present, its numbers form a reasonably close index of the amount of pollution, appears to be proved beyond reasonable cavil. It may safely be said that when the colon bacillus is found in such abundance as to be isolated in a large proportion of cases from 1 c.c. of water, it is generally proof of the presence of serious pollution.

The Problem of Standards. It is obvious, as will be further emphasized in a succeeding chapter, that a sound judgment in regard to the sanitary quality of a particular water supply should be based on a consideration of the facts brought out by a careful sanitary inspection as well as an analytical data. Conclusions based on arbitrary bacteriological standards will never completely fit all cases. Nevertheless, for practical administrative purposes, something in the nature of a standard is often almost essential. The most important standards thus far proposed in the United States must therefore be briefly reviewed.

The first standard of this sort which exerted an important influence was that formulated by the U. S. Public Health Service for the control of water served by common carriers in interstate commerce. (Public Health Reports, XXIX, 2959, Nov. 6, 1914.) The standards imposed at that time are set forth below.

"The following are the maximum limits of permissible bacteriological impurity:

"1. The total number of bacteria developing on standard agar plates, incubated 24 hours at 37 degrees C., shall not exceed 100 per cubic centimeter, provided that the estimate shall be made from not less than two plates, showing such numbers and distribution of colonies as to indicate that the estimate is reliable and accurate.

"2. Not more than one out of five 10-c.c. portions of any sample examined shall show the presence of organisms of the bacillus coli group when tested as follows:

"(*a*) Five 10-c.c. portions of each sample tested shall be planted, each in a fermentation tube containing not less than 30 c.c. of lactose peptone broth. These shall be incubated 48 hours at 36 degrees C. and observed to note gas formation.

"(*b*) From each tube showing gas, more than 5 per cent of the closed arm of fermentation tube, plates shall be made after 48 hours' incubation, upon lactose litmus agar or Endo's medium.

"(*c*) When plate colonies resembling Bact. coli develop upon either of these plate media within 24 hours, a well-isolated characteristic colony shall be fished and transplanted into a lactose-broth fermentation tube, which shall be incubated at 37 degrees C. for 48 hours.

"For the purposes of enforcing any regulations which may be based upon these recommendations, the following may be considered sufficient evidence of the presence of organisms of the bacillus coli group.

"Formation of gas in fermentation tube containing original sample of water (*a*).

"Development of acid-forming colonies on lactose litmus agar plates or bright red colonies on Endo's medium plates, when plates are prepared as directed above under (*b*).

"The formation of gas, occupying 10 per cent or more of closed arm of fermentation tube, in lactose peptone broth fermentation tube inoculated with colony fished from 24-hour lactose litmus agar or Endo medium plate.

"These steps are selected with reference to demonstrating the presence in the samples examined of aërobic lactose-fermenting organisms.

"3. It is recommended as a routine procedure, that in addition to five 10-c.c. portions, one 1-c.c. portion, and one 0.1-c.c. portion of each sample examined be planted in a lactose peptone broth fermentation tube, in order to demonstrate more fully the extent of pollution in grossly polluted samples.

"4. It is recommended that in the above-designated tests the culture media and methods used shall be in accordance with the specifications of the committee on standard methods of water

analysis of the American Public Health Association, as set forth in 'Standard Methods of Water Analysis.' (A. P. H. A., 1912.)"

By this it is seen that water containing more than one colon organism in 50 c.c., or giving an average agar plate count of more than 100 colonies when incubated for 24 hours at 37°, is looked upon as polluted.

This "Treasury Department Standard," as it has commonly been called, has been criticized from many quarters but on the whole has exerted a highly useful influence. It has been modified in various respects since 1914.

In 1922 the Surgeon-General of the United States Public Health Service appointed a series of committees to consider the question of developing a more satisfactory standard of measurement of water supply quality.

The work was divided among three committees, one dealing with the questions of bacteriological examination, another with chemical and physical examination, and the third with sanitary survey problems.

The recommendations of these committees are to be submitted to an Appraisals Committee for final review and adjustment, after which the different sections of the report will be placed before various interested national organizations for discussion and criticism.

The report of the subcommittee on bacteriological examination, as adjusted by the Appraisals Committee, and made public in October, 1923, eliminates the plate count at either 20° or 37°, and bases judgment of the water solely on the presumptive tests followed by certain confirmatory tests. In the discussion of the proposed standard the Committee states:

"It is undoubtedly true, that the 37 degrees C. and 20 degrees C. bacterial counts are of great value and accuracy in routine water analysis for purposes of diagnostic aid and control of purification processes; and the committee wish to record their belief that it would be unwise and unsafe to discard the above indices of water pollution as an auxiliary to *Bact. coli* tests. Simplification of the standard, however, demands the restriction set forth herein to the colon group as the basic unit in the proposed standard."

The new standard, approved by the Advisory Committee which has the matter in charge, reads as follows:

"*A.* Definitions:

1. *Index Organism:* Bact. coli group, as determined in accordance with Standard Methods of the American Public Health Association, current edition, by inoculation in lactose broth fermentation-tube, transplant to endo or eosin-methylene-blue agar plate and inoculation in secondary lactose broth fermentation-tube.

2. *Standard Portion of Water:* Ten cubic centimeters.

3. *Standard Sample of Water:* Five Standard portions of ten cubic centimeters each.

B. Limits of permissible density of bacillus coli-group:

Not more than 10 per cent of all the ten cubic centimeter standard *portions* examined shall show the presence of organisms of the bacillus coli group.

(*a*) When the number of standard *samples* collected is over twenty, not more than five per cent of all the *samples* shall show three or more *positive* tests out of the five 10 c.c. portions comprised in any single sample.

(*b*) When the number of standard samples collected is less than twenty, not more than *one sample* shall show three or more positive tests out of the five 10 c.c. portions.

It is also pointed out that:

"The formation of the *standard* sample does not eliminate in any study the necessity for testing small amounts of water, such as 1, 0.1, 0.01, etc., c.c. for the determination of greater pollutions. It should be emphasized that the range of pollution covered by five to 10 c.c. portions of water is restricted to that in the vicinity of a water bacteriologically safe for drinkiug purposes.

The frequency of collection of standard samples has been given considerable thought. The statistical advantages of frequent sample collections must be balanced against the practical difficulties. With these difficulties in mind, and with due regard to the inadequacy of one or two samples a year, it is recommended that samples of each potable water shall bec ollected in each calendar year in accordance with the requirements of origin and method of treatment of the water."

"In the present standard proposed by the Committee, therefore, two limiting values are suggested. One establishes a limit

for most frequent density of bacilli and the other gives the maximum deviations from the most frequent value which are considered permissible for current practice. The standard tacitly recognizes that such results as one, or two, or three positives out of five 10 c.c. portions may and do occur in the best regulated water supples. The problem confronting the Committee, therefore, is what percentage of excessively high results in a satisfactory water supply may be attributed to chance and what to a definite deterioration of quality which may have dangerous implications?

The first criterion established by the Committee, namely, that " not more than 10 per cent of all the ten cubic centimeter standard portions examined shall show the presence of organisms of the B. coli group," implies a most probable density of one B. coli per 100 c.c. in a water supply, when about 100 portions are tested. In other words, if a water supply is maintained consistently of a bacterial quality equivalent to a concentration of B. coli of one per 100 c.c., then 10 per cent of all 10 c.c. portions thereof examined will be found positive.

On the other hand, chance occurrences have demonstrated and the theory of random sampling would predict that, in this same water of actual constant B. coli density, *some* portions of water tested would contain 3, 5, or 10 B. coli per 100 c.c. or 2, 3, etc., out of five portions of 10 c.c. might be found positive at some time or other. Such variations from constant density are due to errors of simple sampling which may be evaluated, for which allowance should be made in the standard. This is particularly essential, since the standard, in addition to limiting the total percentage of positive portions, must limit also the frequency of *high* results in any single sample, in order to avoid dangerous supplies."

It is obvious that while this method of examination and standardization may be used with large public water supplies in which the bacterial determinations are made regularly and with frequency, it is not so easily applicable to the case of smaller towns, domestic supplies, dairy farms, summer residences, etc., where evaluation of the quality of the water is desirable and where an occasional test or two or three examinations in the course of the year is often the common procedure.

CHAPTER VII

OTHER INTESTINAL BACTERIA WHICH HAVE BEEN USED AS INDICES OF POLLUTION

The Sewage Streptococci. The term "sewage streptococci," as generally used, covers an ill-defined group, including many cocci which do not occur in well-marked chains. Those most commonly found grow feebly on the surface of ordinary nutrient agar, producing faint transparent, rounded colonies, but under semi-anaerobic conditions flourish better, giving a well-marked growth along the gelatin stab and only a small circumscribed film on the surface. They are favored by the presence of the sugars and ferment dextrose and lactose, with the formation of abundant acid but no gas. They are seen under the microscope as cocci, occurring as a rule in pairs, short chains, or irregular groups. They do not show visible growth and do not form indol and nitrite in the standard peptone and nitrate solutions; most of them do not liquefy gelatin, though occasionally forms are found which possess this power. All cocci giving the characteristic growth on agar and strongly fermenting lactose are commonly included as "sewage streptococci."

Although the significance of the streptococci as sewage organisms is not established with the same definiteness which marks our knowledge of the colon group, these forms have been isolated so frequently from polluted sources and so rarely from normal ones that it now seems reasonable to regard their presence as indicative of pollution. Although originally reported by Laws and Andrewes (Laws and Andrewes, 1894), their importance was not emphasized until 1899 and 1900, when Houston (Houston, 1899[b], 1900[b]) laid special stress upon the fact that streptococci and staphylococci seem to be characteristic of sewage and animal waste, the former being, in his opinion, the more truly indicative of dangerous pollution, since they are "readily demonstrable in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination." In six rivers recently extensively sewage-polluted, he found streptococci in from one-tenth to one

ten-thousandth of a c.c. of the water examined, although in some cases the chemical analysis would not have indicated dangerous pollution. On the other hand, eight rivers, not extensively polluted, showed no streptococci in one-tenth of a c.c., although the chemical and the ordinary bacteriological tests gave results which would condemn the waters. Horrocks (Horrocks, 1901) found these organisms in great abundance in sewage and in waters which were known to be sewage-polluted, but which contained no traces of *Bact. coli*. He found by experiment that *Bact. coli* gradually disappeared from specimens of sewage kept in the dark at the temperature of an outside veranda, while the commonest forms which persisted were varieties of streptococci and staphylococci.

In America attention was first called to these organisms by Hunnewell and one of us (Winslow and Hunnewell, 1902[a]), and the same authors later (Winslow and Hunnewell, 1902[b]) recorded the isolation of streptococci from 25 out of 50 samples of polluted waters. Gage (Gage, 1902), from the Lawrence Experiment Station, has reported the organisms present in the sewage of that city, while Prescott (1902[b]) has shown that they are abundant in fæcal matter and often overgrow *Bact. coli* in a few hours when inoculations are made from such material into dextrose broth. In the monograph of Le Gros (Le Gros, 1902) of the many streptococci described, all without exception were isolated, either from the body or from sewage. Baker and one of us (Prescott and Baker, 1904) found these organisms present in each of 50 samples of polluted waters. On the other hand, in the study of 259 samples of presumably unpolluted waters, by the method of direct plating, Nibecker and one of the authors (Winslow and Nibecker, 1903), found streptococci in only one sample. Clemesha (1912[a]) finds that streptococci in India are present in .0001 or .00001 gm. of fæces, but are rare in waters unless very grossly polluted. In a series of bottle experiments in the laboratory and in the study of an artificially polluted tank outdoors he showed that they disappear very rapidly in water, within 2 or 3days at the most. Gordon (1904) showed that certain streptococci are abundant in normal saliva and are found in air which has been exposed to human pollution but not in normal air. On the whole there can be no doubt of the fact that streptococci occur on the surfaces of the human and animal body more commonly than anywhere else in nature.

Isolation of Sewage Streptococci. The isolation of these organisms either from plates or liquid cultures is easy. On the lactose-agar plate, made directly from a polluted water, the colonies of the streptococci may generally be distinguished from those of other acid-formers by their small size, compact structure, and deep-red color, which is permanent, never changing to blue at a later period of incubation. Developing somewhat slowly, however, they may be overlooked if present only in small numbers. In the dextrose-broth tube, streptococci will generally appear in abundance after a suitable period of incubation. Prescott and Baker, in the work above mentioned, found that with mixtures of *Bact. coli* and streptococci in which the initial ratios of the latter to the former varied from 1 : 94 to 208 : 1, the colon bacilli developed rapidly during the early part of the experiment, reaching a maximum after about 14 hours, and then diminishing rapidly. The streptococci first became apparent after 10 to 15

RELATIVE GROWTH OF BACT. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH

(Prescott and Baker, 1904)

Sample Number			1	2	3	4	5	6	7	8	9	10
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			4	10	9	5	8	55	35	460	1250	105
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	11 hrs.	Bact. coli	0	20	68	200	185	400	130	332	420	410
		Strept	0	0	0	0	0	0	0	0	0	0
	16 hrs.	Bact. coli	200	76	130	270	220	210	140	420	285	410
		Strept	40	25	20	10	45	30	20	210	75	145
	23 hrs.	Bact. coli	280	150	385	370	300	570	200	405	320	300
		Strept	140	85	280	170	300	1700	110	350	370	350
	39 hrs.	Bact. coli	0	0	25	110	0	210	20	24	105	
		Strept	474	420	480	300	390	170	400	105	250	
	63 hrs.	Bact. coli	0	0	0	0	0	12	8	0	0	0
		Strept	2	0	0	45	1	2	45	150	86	170
First gas noted after (hrs)			10	10	9	9	10	8	10	6	6	8

hours and reached their maximum after 20 to 60 hours, according to the number originally present.

Applying the same method to polluted waters, similar periodic changes were observed; nearly pure cultures of *Bact. coli* were first obtained, then the gradual displacement of one form by the other took place, and at length the streptococci were present either in pure culture or in great predominance as shown by the accompanying tables. The samples of water were plated directly upon litmus lactose agar and the plates were incubated at 37° for 24 hours, when the red colonies were counted. At the time of plating, 1 c.c. from each sample was also inoculated into dextrose broth in fermentation tubes, which were likewise incubated at 37°. After various periods, as indicated by the tables below, the tubes were shaken thoroughly and 1 c.c. of the contents withdrawn. This was diluted (generally 1–1,000,000,) with sterile water, plated on litmus lactose agar in the usual way, and incubated for 24 hours. The colonies of *Bact. coli* and streptococci were

RELATIVE GROWTH OF BACT. COLI AND SEWAGE STREPTOCOCCI FROM POLLUTED WATERS IN DEXTROSE BROTH

(Prescott and Baker, 1904)

Sample Number			18	19	20	21	22	23	24	25
Red colonies developing from 1 c.c. of original sample on litmus lactose agar			1	150	25	30	50	170	200	30
Number found, in millions per cubic centimeter, after growth in dextrose broth for various periods	7 hrs.	Bact. coli	.02	...	...	.01	.04	.12	.55	1.6
		Strept.	0	...	...	0	0	0	0	0
	17 hrs.	Bact. coli	266	100	88	350	510	380	330	160
		Strept.	150	0	40	140	240	128	80	220
	27 hrs.	Bact. coli	520	610	72	700	1000	740	100	300
		Strept.	800	860	670	1080	2500	4380	...	3900
	40 hrs.	Bact. coli	0	0	10	22	36	7	7	
		Strept.	252	330	260	22	66	60	52	
	52 hrs.	Bact. coli	10	16	38	20	70	35	10	27
		Strept.	40	16	3.8	31	41	25	10	30

distinguished microscopically, and by difference in color and general characters.

The successive growth of these two intestinal groups in the same dextrose-broth tube suggests the following method for the detection of both *Bact. coli* and sewage streptococci.

Inoculate the desired quantity of water, preferably 1 c.c., into dextrose broth, in a fermentation tube, and incubate at 37°. After a few hours' incubation examine the cultures for gas. Within 2 or 3 hours after gas formation is first evident, plate from the broth in litmus lactose agar, incubating for 12 to 18 hours at 37°. If at the end of this time no acid-producing colonies are found, it is probably safe to assume that there were no colon bacilli present. On the other hand, if red colonies are developed, these must be further examined by the regular diagnostic tests for *Bact. coli*. After the first plating from the dextrose broth, replace the fermentation tube in the incubator and allow it to remain for 24 to 36 hours, then plate again on litmus lactose agar. This plating should give a nearly pure culture of streptococci if these organisms were originally present in the water.

Streptococci as Indicators of Recent Pollution. The comparative relation of the streptococci and the colon bacilli to sewage pollution is still somewhat uncertain. Houston (Houston, 1900) held that the former microbes imply "animal pollution of extremely recent and therefore specially dangerous kind," and Clemesha's experiments led to the same conclusion. Horrocks (Horrocks, 1901), on the other hand, maintains, largely on the strength of certain experiments with stored sewage, that the streptococci persist after colon bacilli have disappeared and indicate contamination with old sewage which is not necessarily dangerous. These discordant results are probably to be explained by the different media in which the viability of the bacteria was compared. It seems likely that in sewage where there is a large amount of organic food material present the streptococci may kill out the colon bacilli as they do in the fermentation tube, and as we know they frequently do in shellfish. This would explain Horrocks' results. On the other hand, there is good evidence that the streptococci are less resistant than *Bact. coli* to the unfavorable conditions which exist in water of ordinary organic purity. In waters of potable character *Bact. coli* is frequently present without the streptococcus; and a negative test for streptococci has little

significance. A positive test, on the other hand, furnishes valuable confirmatory evidence of pollution. This evidence is of course of special importance when through the activity of the streptococci themselves, or from any other cause the colon isolation has yielded an erroneous negative result.

The English Committee appointed to consider the standardization of methods for the bacterioscopic examination of water (1904) by a majority vote recommended the enumeration of streptococci, as a routine procedure in sanitary water analysis, but in this country the Committee on Standard Methods of Water Analysis (1912) has concluded that "the information afforded by the occurrence of these organisms seems to be of less value than in the case of *Bact. coli* and it is believed that for the present at least, the streptococcus test is of subordinate importance." Later reports of this Committee make no mention of this examination.

Savage and Read (1917), more recently, examined a considerable series of waters for colon bacilli and streptococci. To detect the latter they added the water sample to glucose-neutral-red broth and made a direct microscopic examination for chains after 40–48 hours at 37°. They find a general correspondence between the results of the two tests as indicated below for a group of 974 surface waters.

	Bact. coli	No samples	Percentage of each subgroup containing streptococci in			
Subgroup			0.1 or 1 c.c.	10 c.c.	30 c.c.	Not in 40 c.c
A	In 0.1 or 1 c.c.	443	53	32	9	6
B	In 10 c.c.	249	19	35	24	22
C	Not in 10 c.c.	282	3	18	22	56

It will be noted, however, that a number of samples containing considerable numbers of *Bact. coli* were negative for streptococci. In general the streptococcus test seems less delicate than the colon test and adds nothing that the colon test does not give.

Savage and Wood (1918) in an experimental study of the viability of streptococci in water found that they died out in parallel with the colon bacilli, although a trifle more rapidly.

Use of the Streptococci to Distinguish between Human and Animal Pollution. There seems some reason to hope that the streptococci may prove of assistance in the important task of differentiating between human and animal pollution, a task in which all other tests have so far failed. Unlike the colon bacilli, streptococci from the intestines of cattle and men appear to belong to distinct types. The recognition of this fact we owe primarily to Gordon (1905), who made an elaborate study of the fermentative power of the streptococci in a long series of carbohydrate media. His work and that of Houston (Houston, 1904; Houston, 1905[a], Houston, 1905[b]) have made it clear that the streptococci of the herbivora differ from those found in the human body in their low fermentative power. In their review of the genus, Andrewes and Horder (1906) describe the type characteristic of the herbivora under the name, *Str. equinus*, and define it by its failure to ferment lactose, raffinose, inulin or mannite, or to reduce neutral red. Five other types are described from the human mouth and intestine; all of them ferment lactose, and most reduce neutral red and ferment raffinose. The commonest intestinal form clots milk, reduces neutral red and ferments saccharose, salicin, coniferin and mannite. The specific types of the genus Streptococcus grade into each other by almost imperceptible degrees, and streptococci fermenting lactose and raffinose and reducing neutral red are sometimes found in bovine fæces; but the studies made in this country by Winslow and Palmer (1910) confirm the conclusions of the English observers that there are specific differences between the streptococci of the human, bovine, and equine intestines. The most important of these results are indicated in the table below:

COMPARATIVE FERMENTATIVE POWER OF STREPTOCOCCI FROM THE HORSE, THE COW, AND MAN

(Winslow and Palmer, 1910)

Streptococci	Percentage of Positive Results (300 Strains)		
	Lactose	Raffinose	Mannite
Human	62	6	28
Equine	8	4	2
Bovine	52	28	6

The rarity of lactose-fermenting streptococci in the horse makes it probable that this group can be used for distinguishing pollution by street washings from that due to domestic sewage; and the fact that a considerably larger proportion of human strains attack mannite and a considerably larger proportion of bovine strains ferment raffinose should make it possible to use the ratio between results in these two media to distinguish between the wash from pastures and cultivated land and sewage. Clemesha (1912[a]) in India found that both human and bovine fæcal streptococci fermented raffinose, saccharose, and salicin, but not mannite. Fuller and Armstrong, however, confirm the results obtained by Winslow and Palmer, finding that lactose-fermenting streptococci of any kind are rare in horse dung, that the streptococci of bovine fæces characteristically ferment lactose and raffinose, while the fermentation of lactose and mannite is usual among human fæcal strains. The results of these workers were more consistent than those tabulated above, 65 per cent of the human strains being of the *Str. fæcalis* type (mannite-positive), while 64 per cent of the bovine strains were of the *Str. salivarius* (raffinose-positive) type. Rogers and Dahlberg (1914) also found that streptococci from bovine fæces typically ferment raffinose and not mannite, while those from the mouth of the cow are frequently of the *Str. salivarius* type. Among the hemolytic streptococci, those of bovine origin appear to be able to hydrolyze hippuric acid while those of human origin do not (Ayers and Rupp, 1922). This differential test does not, however, apply to the non-hemolyzing types.

A special use was made of the streptococcus test by Pettibone, Bogart and Clark (1916), who employed it for the detection of mouth pollution in an examination of bubble fountains at the University of Wisconsin.

The Anaerobic Spore-forming Bacilli. The English bacteriologists have ascribed much importance as indicators of sewage pollution to another group of organisms, the anaerobic spore-forming bacilli, of which the form described as *Bact. aerogenes-capsulatus* (Welch and Nuttall, 1892) and now called *Bact. welchii* (or *Cl. welchii*) and the form isolated by Klein (Klein, 1898; Klein, 1899) in 1895 in the course of an epidemic of diarrhœa at St. Bartholomew's Hospital, described under the name of *Bact. enteritidis sporogenes* (now called *Bact. sporogenes*) are types. (Also classed by Levine as *Cl. welchii*).

The procedure originally described by Klein for isolating *Bact. sporogenes* is as follows: a portion of the sample to be examined is added to a tube of sterile milk, which is then heated to 80° C. for 10 minutes to destroy vegetative cells. The milk is next cooled and incubated under anaerobic conditions, which may be accomplished conveniently by Wright's method. A tight plug of cotton is forced a quarter way down the test-tube, the space above is loosely filled with pyrogallic acid, a few drops of a strong solution of caustic potash are added, and the tube is tightly closed with a rubber stopper. After 18 to 36 hours at 37° the appearance of the tube will be characteristic if the *Bact. sporogenes* is present. "The cream is torn or altogether dissociated by the development of gas, so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein, enclosing a number of gas-bubbles. The main portion of the tube formerly occupied by the milk now contains a colorless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened, the whey has a smell of butyric acid and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless."

Since this organism is not present in very large numbers, even in sewage, the test of a water-supply must be made with large samples, and the concentration of at least 2000 c.c. of water by filtration through a Pasteur filter is recommended by Horrocks as a necessary prelude (Horrocks, 1901). The Committee on Standard Methods of Water Analysis (1912) recommended the following enrichment procedure for the isolation of *Bact. sporogenes*. Various dilutions of the water to be tested are incubated in fermentation tubes containing liver broth for 24 hours at 37°. If *Bact. sporogenes* is present gas will be evolved and a characteristic "vile odor" will be produced. If this reaction is obtained the contents of each positive tube are transferred to an Erlenmeyer flask or large test-tube and heated at 80° C. for 10 minutes to destroy vegetative cells. One c.c. of broth containing sediment is withdrawn from the bottom of each flask and enriched once more in a fresh liver broth tube. *Bact. sporogenes* will now usually be present in pure culture showing large sluggishly motile bacilli containing spores. A gelatin stab culture made from these 24-hour liver broth tubes will show after 48 hours' incubation at 20° a distinct liquefying anaerobic growth beginning about 2

cm. below the surface with gas bubbles at the top of the liquefied area. In order to obtain absolutely pure cultures it is necessary to fish from liver broth tubes only 3–5 hours old as only young vegetative cells will grow on plates. Transplants from the closed arm of such tubes will grow on dextrose liver agar plates incubated under anaerobic conditions.

Another method for isolating *Bact. sporogenes* suggested by Wells is simpler than the foregoing. He recommends the use of freshly-boiled lactose broth tubes (heated in the Arnold Sterilizer) brought to a temperature of 70 degrees C., at which temperature the inoculation is made. The inoculated tubes are then kept at 70 degrees C. for 10 minutes and subsequently incubated at 37 degrees C. By this means the spore forms of *Bact. sporogenes* develop into vegetative cells in the course of 24 hours and give rise to a rapid evolution of gas. Aerobic plates made from these tubes will generally give no growth. On the other hand, if *Bact. coli* were present, gas would ordinarily be produced in 18 hours and aerobic plates, either on litmus agar or Endo medium would show an abundance of colonies.

The organisms of the *Bact. sporogenes* group are large stout bacilli often occurring in chains. They liquefy gelatin vigorously and on agar produce fine discrete gray colonies. They vigorously ferment dextrose, lactose and saccharose, producing acid and gas, and in sugar agar each colony will be marked by one or more gas bubbles surrounded by a delicate whitish fringe.

Much work was done during and since the Great War in regard to the systematic relationships of this group of anaerobic sporeformers which proved to be of such importance in connection with wound infections. Space forbids an analysis of this material which is well reviewed, from the standpoint of the water bacteriologist, by Levine (1921).

Significance of the Anaerobes in Water Analysis. The researches of Klein and Houston (Klein and Houston, 1898, 1899) have shown that the *Bact. sporogenes* occurs in English sewage in numbers varying from 30 to 2200 per c.c. and that it is often absent in considerable volumes of ordinary surface waters. In Boston sewage it may usually be isolated from .01 or .001 of a c.c. (Winslow and Belcher, 1904). Since the spores of an anaerobic bacillus may persist for an indefinite period in polluted waters,

their presence need not necessarily indicate recent or dangerous pollution.

Levine (1921) believes that the general use of the anaerobic spore formers as indices of pollution is undesirable, on account of their extreme resistance, their abundance in animal manure, in decomposing organic matter and in the soil and the lack of correlation between the numbers of these organisms present in water and the results of the sanitary survey. He cites with approval Cumming's statement that "unlike *Bact. coli* which varies many thousand per cent, from several hundred per c.c. to less than 1 in 10 c.c., according to the intensity of pollution, these spores were found often in the best river water in 10 c.c. and seldom showed an average much above 4 or 5 per c.c. . . . Their number furnishes no clue to the degree of pollution and purification as does the number of *Bact. coli*."

Larner (1922) on the other hand describes two epidemics of mild intestinal disease which occurred in Montclair, N. J., in the winter months of 1918 and 1921, respectively, and attributes them to pollution of the public water supply. On both occasions anaerobic spore formers were present and similar organisms were isolated in considerable numbers from the fæces of several persons suffering from the disease in question. Larner believes that harmfulness of *Bact. welchii* is very much an open question and that waters containing this organism in large numbers should be regarded with grave suspicion.

Vincent (1907) and other French observers consider the determination of the total number of anaerobic bacteria as significant, since the decomposition of organic matter is accompanied by anaerobic growth. It is not claimed, however, that bacteria of this type are characteristic of animal more than of vegetable decompositions, and the total anaerobic count apparently adds nothing of importance to the information gained by the ordinary gelatin plate method. The property of liquefaction was formerly believed to be of significance, inasmuch as the liquefying bacteria were regarded as indicative of pollution. This position is, however, no longer tenable, since many bacteria, typical of the purest waters, may cause liquefaction.

Except in rare instances it is now held that the chief interest of the water bacteriologist in these spore forms is due to their interference with the presumptive tests.

The Isolation of the Typhoid Bacillus from Water. In view of the fact that the typhoid bacillus is the organism most to be feared in a water supply it might naturally be asked why we do not use this organism itself as an index of pollution. The answer is that it can be found in water so rarely as to be of no practical value for such a purpose. There are, it is true, a number of instances on record in which this organism has undoubtedly been isolated from polluted water, as by Kübler and Neufeld (Kübler and Neufeld, 1899), who examined a farmhouse well at Neumark in 1899, and Fischer and Flatau (Fischer and Flatau, 1901), who discovered an organism responding to a most exhaustive series of tests for the typhoid bacillus in a well at Rellingen in 1901. In these cases the water was directly plated upon Elsner's medium or phenolated gelatin with no preliminary process of enrichment. Willson (Willson, 1905) summarized the instances in which the typhoid bacillus had been isolated from infected drinking water, up to 1905, and included, in addition to the above-mentioned cases, the following:

1. By Lösener, in 1895, from the Berlin water supply.
2. By Conradi, in 1902, from a well at Pecs in Hungary, by use of carbol gelatin plates.
3. By Jaksch and Rau, in 1904, from the water supply of Prague, and also from the river Moldau, by caffein-nutrose crystal violet agar.
4. By Ströszner, in 1904, from a well near Budapest, by the same method.

Several other instances in which the isolated organisms gave positive agglutination tests, as well as the usual cultural reactions, are also cited by Willson.

Since 1905 a number of successful isolations of the typhoid bacillus have been reported in America. An organism obtained from the water-supply of Scranton, Pa., in 1907, by simple enrichment in Parietti bouillon, was identified as the typhoid bacillus by Prof. Fox after a very careful series of tests with immune sera (Pennsylvania, 1908). The most important results have been achieved, however, by Jackson with lactose bile enrichment and subsequent plating on Hesse agar. He reports the isolation of *Bact. typhosum* from 10 c.c. samples of the Grass River at Canton, N. Y., and of a pond and stream at Hastings, N. Y., (both used as sources of water-supply) and from two 1 c.c. samples

of the Hudson River near Hastings at the time of the typhoid epidemic there (Jackson and Melia, 1909). Stokes and Hachtel (1910) by the same method found organisms corresponding to typhoid in their general cultural reactions in four samples of surface-waters (two of them from an impounding reservoir of the Baltimore supply), in the sediment of a school well supposed to have caused typhoid fever, in a sewage-polluted stream and in two samples of market oysters. These organisms agglutinated with the blood of typhoid patients in 1/50 and 1/100 dilutions, but with an immune serum producing agglutination with a standard laboratory typhoid culture in dilution of 1/25,000 these water organisms would only agglutinate in dilutions of 1/250 or 1/500. Their identity must therefore be regarded as somewhat doubtful. The same authors (Stokes and Hachtel, 1912) have reported the isolation of the typhoid bacillus from the water in the neighborhood of a polluted oyster bed. In recent years Pettersson (1919) reports finding the typhoid bacillus in a polluted well at Visby by the iron sulphate precipitation method. Geiger, MacMillan and Gillespie (1917) even isolated the typhoid bacillus by direct plating on litmus lactose agar, from a brook associated with a typhoid epidemic at a labor camp in California. The water of the brook in question was however so heavily polluted as to be more properly designated as "dilute sewage."

The search for the typhoid bacillus is usually suggested when an outbreak of the disease has cast strong suspicion upon some definite source of water-supply. By the time an epidemic manifests itself, however, the period of the original infection is long past, and the chances are good that any of the specific bacilli once present will have disappeared. While elaborate experiments have shown that *Bact. typhosum* may persist in sterilized water for upwards of 2 months and in unsterilized water from 3 days to several weeks, the number of the organisms present is always very rapidly reduced. Even in highly polluted water their number is proportionately small, as is well shown by the experiments of Laws and Andrewes (Laws and Andrewes, 1894) who entirely failed to isolate the typhoid bacillus from the sewage of London and found only two colonies of the organism on a long series of plates made from the sewage of a hospital containing forty typhoid patients. So Wathelet (Wathelet, 1895) found that of 600 colonies isolated from typhoid stools and having the appearance characteristic of

Bact. coli and *Bact. typhosum* only 10 belonged to the latter species.

Epidemiological evidence confirms these results and indicates that the number of typhoid bacilli even in polluted water probably is never very great, while the fate of Lowell and Lawrence in 1890–91 and the more recent epidemics at Butler, Pa., and Ithaca, N. Y., demonstrate that even a small number of virulent organisms can bring about an almost wholesale infection. Indeed, if the virulent organism were as abundant as some results would indicate (Remlinger and Schneider, 1897), the human race would long since have been exterminated. A negative result in testing for typhoid bacilli has no significance and there is danger that it may be misinterpreted if the fact that it has been made comes to public knowledge. In spite of this danger, however, and in spite of the laborious and time-consuming nature of the process, the increasingly large number of positive isolations in recent years indicates that it is well worth trying in cases of special importance. The search for the typhoid bacillus should of course never supersede the examination for colon bacilli, since the latter are so much more numerous in water and so much more easily identified. Because of these facts, colon bacilli will continue to be our best index of pollution, while the isolation of the typhoid bacillus may be attempted in certain special cases as contributory evidence, but generally with the probability of failure.

Methods for Isolating the Typhoid Bacillus from Water. The methods used for the isolation of the typhoid bacillus from water fall in general into three main groups; (*a*) direct isolation on differential solid media; (*b*) isolation as above after preliminary cultivation in a selective enrichment medium; (*c*) isolation, with or without enrichment, preceded by physical concentration produced by agglutination or chemical precipitation.

We have seen that direct plating on phenolated gelatin or litmus-lactose-agar has proved a successful procedure in certain instances. Drigalski-Conradi agar (Drigalski and Conradi, 1902), Endo agar (Endo, 1904) malachite-green agar (Loeffler, 1903 and 1906; Lentz and Tietz, 1903 and 1905; Doebert, 1900, Nowack, 1905), and brilliant-green agar (Torrey, 1913; Krumwiede and Pratt, 1914) have been extensively used for this purpose, the last being probably the most promising. All of these media depend on the theory that the typhoid bacillus is more resistant than *Bact.*

coli to the dyes present in the media. The semi-solid media of Hiss and Hesse (Hiss, 1902; Jackson and Melia, 1909; Jackson, 1909; Stokes and Hachtel, 1912) depend on the other hand on the greater motility of *Bact. typhosum* and its consequent tendency to form branching colonies and turbid zones on such media.

Among the more important enrichment media, supposed to favor the growth of *Bact. typhosum* at the expense of *Bact. coli* are phenol broth (Parietti, 1890; Hankin, 1899) media containing caffein (Roth, 1903; Hoffman and Ficker, 1904); Kloumann, 1904; Willson, 1905), lactose bile (Jackson and Melia, 1909) and brilliant-green broth.

A physical concentration of the typhoid bacilli precedes enrichment or isolation in the procedure recommended by many authors. Klein accomplished this by passing the water through a Berkefeld filter. Other workers have made use of agglutination or chemical precipitation for the same purpose.

The phenomenon of agglutination was made the basis of a method of isolating *Bact. typhosum* from water by Adami and Chopin (Adami and Chopin, 1904). Two-liter samples of the water were collected in sterilized bottles (Winchester quarts), and to each was added 20 c.c. of 1 per cent glucose broth. The sample was incubated for 18 to 24 hours at 37° C., after which 10 c.c. portions were withdrawn and placed in long, narrow test tubes. To each of these tubes enough typhoid serum of known potency was added to make a regularly graded series, 1–50, 1–100, 1–150, and 1–200. The probable presence of the typhoid bacillus was manifest by the formation of flocculi within a quarter of an hour, and agglutination was complete in from 2 to 5 hours.

The tube having the greatest dilution in which agglutination was apparent was then examined by breaking off the lower end, containing the precipitate, washing the sediment two or three times with sterile water after removing the clear supernatant liquid, and allowing the bacteria to settle again. The organisms remaining were plated upon various media, and examined biochemically to determine the true character of the suspected colonies. It was found that a dilution of 1 to 60 was the highest which could be used with the organisms examined, and it is therefore probable that high dilutions (greater than 1–60) cannot be successfully used.

Investigation of an organism isolated by this method was made

by Klotz (1904), who found the culture to be not a typical *Bact. typhosum*, but a form showing certain points of similarity to both *Bact. typhosum* and to *Bact. coli*, and probably intermediate between them. Frost (1910) isolated a bacillus of the Proteus group from filtered Potomac water which agglutinated with typhoid serum in high dilutions. As Klotz points out, therefore, it is evident that even when a positive result is obtained with a relatively high dilution of typhoid serum, the action may by no means be absolutely specific.

Schepilewski (Schepilewski, 1903) and Altschuler (Altschuler, 1903) have also used agglutination as a means of precipitating the bacteria after enrichment cultivation in broth. The former incubated the culture at 37° for 24 hours, then added a serum of high potency, allowed the mixture to stand for 2 to 3 hours, and then centrifuged. The supernatant liquid was removed, and the mass of agglutinated cells broken up by shaking with glass beads and salt solution. Upon plating upon litmus lactose agar the organisms could be detected. In this way positive isolation was made from water containing 1 loopful of a broth culture in 50 liters of water. Altschuler's method of enrichment was essentially like that of Schepilewski. From the surface of the culture developed at 37°, 10 c.c. were removed to a tapering tube provided with a rubber tube at the bottom. Serum was added in the proportion of one part in 50, the culture agitated to release entangled non-agglutinated bacilli and the sediment run into a tube containing 1 per cent peptone and 0.5 per cent salt. The agglutinated mass was broken up by shaking with sand, and the culture incubated at 37° for 24 hours, then plated on Drigalski-Conradi plates. The organism was isolated from dilute suspensions in water (150 in 1 liter) and also from the fæces of a typhoid patient from which other methods gave negative results.

A number of methods for concentrating typhoid bacilli in water by chemical precipitation have been tested experimentally, with some degree of promise. Vallet (Vallet, 1901) was the first to employ this principle, and made use of sodium hyposulphite and lead acetate. The mixture was then centrifuged and the precipitate dissolved in more hyposulphite. The clear solution was then plated.

Schüder (Schüder, 1903) observed that the lead salt reacted harmfully upon the bacteria. and that the hyposulphite should

be in excess. In his experiments water was allowed to stand in tall jars for 24 hours. To 2 liters of infected water, 20 c.c. of a 7.75 per cent solution of sodium hyposulphite was added, and after thorough mixing 20 c.c. of a 10 per cent solution of lead nitrate. The precipitate, after 20 to 24 hours, was treated with 14 c.c. of saturated sodium hyposulphite solution and shaken. From the clear solution 0.2 to 0.5 c.c. portions were streaked upon Drigalski-Conradi plates which were then incubated at 37° for 24 hours. Ficker (Ficker, 1904) modified the process still more by using ferric sulphate, and dissolved the precipitate with neutral potassium tartrate. The final solution was then plated on Drigalski-Conradi medium. Ficker claimed that this method gives excellent results, 97–98 per cent of the typhoid bacteria being carried down with the precipitate.

Müller (Müller, 1905), in comparing different precipitation methods, adopted ferric oxychloride as the most suitable precipitant, because of its quicker and less destructive action. Willson (Willson, 1905) suggested the use of alum as a precipitant. He added 0.5 gr. alum per liter of water examined. The mixture was then centrifuged, and the precipitate suspended in a small amount of water and plated on Drigalski-Conradi medium. Nieter (Nieter, 1906) made 20 parallel experiments, using very pure water infected with typhoid bacilli in varying numbers. By precipitating with ferric sulphate and sodium hydrate, centrifuging, and then filtering through a sterile filter he obtained small numbers of bacteria. Using iron oxychloride as the precipitant, he confirmed the results of Müller. By plating on malachite green agar he was often able to get positive results when the Drigalski-Conradi medium failed.

By use of a combination of enrichment and chemical precipitation, Ditthorn and Gildemeister (Ditthorn and Gildemeister, 1906) isolated the typhoid bacillus from enormous artificial dilutions in water. In the typhoid fever epidemic in Posen, in 1906, it was found that the bile of those dying from the disease contained nearly pure cultures of typhoid bacilli. This led the authors mentioned to use bile and bile agar as enrichment media. After precipitating by Müller's method, the whole of the precipitate was added to 100 c.c. sterile ox bile and grown at 37° for 24 hours, after which time 1 c.c. portions were plated. With extreme dilutions it was found desirable to incubate for 48 to 72 hours.

The results were unsatisfactory in the presence of large numbers of water bacteria. It is also pointed out that the iron oxychloride is bactericidal in 48 hours.

Drigalski (Drigalski, 1906) has suggested the separation of *Bact. typhosum* from other bacteria in water through its greater motility. He succeeded in isolating typhoid bacilli from two springs by the following method: 5 to 10 liters of water were allowed to stand one to two days in tall milk cans at room temperature. Samples were taken from the surface and plated on litmus-lactose agar (Drigalski-Conradi medium), the amount of water to be used varying with the contamination.

Starkey (1906) has suggested the use of an apparatus consisting of a piece of glass tubing bent so as to give four successive connected loops. This is filled with phenol broth, inoculated at one end, and incubated anærobically. The more actively motile bacilli find their way to the fourth loop from which they may be isolated by plating.

The methods of examining water for *Bact. typhosum* may be conveniently summarized as follows:

- Examination of water for typhoid bacilli
  - 1. Physical concentration
    - *a.* By filtration
    - *b.* By agglutination
    - *c.* By chemical precipitation
      - Schüder's process
      - Fischer's process
      - Willson's process
      - Müller's process
  - 2. Enrichment
    - *a.* Parietti's carbol broth
    - *b.* Jackson's lactose bile
    - *c.* Hoffmann and Ficker's caffein process
    - *d.* Brilliant green broth
  - 3. Isolation
    - *a.* Elsner's gelatin medium
    - *b.* Endo's medium
    - *c.* Loeffler's malachite green medium
    - *d.* Brilliant green agar
    - *e.* Drigalski-Conradi agar
    - *f.* Hiss's medium
    - *g.* Hesse's medium
  - 4. Identification
    - *a.* Morphological and cultural characters
    - *b.* Agglutination

Of the comparative advantages of these methods it is unwise to claim to speak with finality. Up to the present time the use of caffein and lactose bile has apparently been followed by the best results, and it seems likely that of the precipitation methods that employing the oxychloride of iron is the best. The successful use of malachite green and brilliant green in the isolation of typhoid and paratyphoid bacilli from fæces suggests that the more general adoption of these media in water examinations would possibly prove fruitful.

When an organism resembling the typhoid bacillus has at last been isolated its identity must of course be established by exhaustive biochemical tests and confirmed by agglutination. This procedure will rarely be adopted by the water bacteriologist. In those cases where it is attempted, or where search is to be made for the dysentery and paratyphoid organisms, recourse may be had to the Russell double-sugar agar (Russell, 1911), the Krumwiede triple-sugar agar (Krumweide, Pratt and McWilliams, 1916; Krumwiede and Kohn, 1917), the saccharose-mannitol agar of Kendall (Kendall, 1919), lead acetate agar and the other special media which have been employed for this purpose. The student is referred to the original articles cited above or to standard textbooks of medical bacteriology for the exact composition of the media and the technique employed. The following table

REACTIONS OF THE VARIOUS ORGANISMS ON DOUBLE SUGARS

	Russell 0.1% Glucose 1% Lactose		Kendall 0.1% Mannitol 1% Saccharose	
	Butt	Slant	Butt	Slant
Bact. alcaligenes	c	c	c	c
Bact. dysenteriae Shiga	r	c	c	c
Bact. dysenteriae Flexner	r	c	r	c
Bact. dysenteriae Strong	r	c	r	r
Bact. typhosum	r	c	r	c
Bact. No. 1 Morgan	g	c	c	c
Bact. paratyphosum A	g	c	g	c
Bact. paratyphosum B	g	c	g	c
Bact. coli A	g	r	g	c
Bact. coli B	g	r	g	r
Bact. proteus	g	c	g	r
Bact. cholerae	r	r	r	r
Streptococcus	r	r	r	r

r = acid, c = color unchanged, g = gas and acid

by Kendall may prove of interest as showing the comparative reactions of the organisms for the identification of which these media may be employed.

The Colon-Typhoid Intermediates. It will be noted by reference to the table cited at the beginning of Chapter V that there is an important group of organisms whose reactions are in general intermediate between those of *Bact. coli* and *Bact. typhosum*. This is the hog cholera group, or the Gärtner group, as Durham (1898) called it. As defined by him, it differed from the typhoid group by gas formation in dextrose, and from the colon group by the production of a final alkaline reaction in milk. It includes the Gärtner bacillus (*Bact. enteritidis*), the hog cholera bacillus (*Bact. suipestifer*), and the paratyphoid bacilli. Some of these forms, the paratyphoid bacilli, for example, and *Bact. enteritidis* (isolated in cases of meat poisoning), produce intestinal disease in man.

Starkey (1909 and 1911) believes that all organisms giving the general reactions of the Gärtner and paratyphoid groups are significant and warrant the condemnation of a water supply. Kligler (1919) has been successful in isolating non-lactose-fermenting bacilli of the colon-typhoid intermediate group from a series of polluted wells and subsoils. In wells paratyphoid-like forms, in subsoils dysentery-like forms, were most abundant. He believes that the former were derived chiefly from animal excrement washed in at the surface of the wells, the latter from human excrements carried through the soil from privies. It is apparent that a study of these non-lactose-fermenting intermediates may prove of considerable importance to the sanitarian in special instances. The difficulty, however, is that while non-acid-forming bacteria of this general type are sometimes found in fæces, they are also found in other habitats, and they are less abundant proportionately, in polluted than in stored and safer waters. If true dysentery and paratyphoid bacilli can be isolated and identified by serum reactions it is, of course, highly important. Houston (1911), however, has carefully tested the method suggested by Starkey (1906) for isolating these forms and found that it gave negative results even with a water artificially infected with about 14 typhoid bacilli and 21 Gärtner bacilli per c.c. In his own studies Houston reports that in the examination of 13,442 microbes from polluted river water he found only one member of the Gärtner

group; and in another study of 20,771 colonies he found only 2 typhoid-like forms.

Isolation of the Cholera Spirillum. The isolation of the cholera spirillum from water can probably be accomplished with somewhat less difficulty than is encountered in the case of *Bact. typhosum*. Schottelius (Schottelius, 1885) was the first to point out the necessity for growing this organism in an alkaline medium, and Loeffler (Loeffler, 1893) found that its isolation from water could be successfully accomplished by adding 10 c.c. of alkaline pepton broth to 200 c.c. of the infected water and incubating for 24 hours at 37 degrees, when the organism could be found at the surface of the medium.

Somewhat earlier than this Dunham (Dunham, 1887) had made a special study of the chemical reactions of the cholera spirillum and found that the organism would grow abundantly in a solution containing 1 per cent peptone and 0.5 per cent salt (Dunham's solution), producing the "cholera-red or nitroso-indol reaction." This medium was brought into practical use by Dunbar (Dunbar, 1892), who succeeded in isolating the organisms from the water of the Elbe in 1892, during the cholera epidemic at Hamburg.

Koch (Koch, 1893) prescribed the following method for the isolation of the organism from water:

To 100 c.c. of the water to be examined is added 1 per cent pepton and 1 per cent salt. The mixture is then incubated at 37 degrees. After intervals of 10, 15, and 20 hours the solution is examined microscopically for comma-shaped organisms, and agar plate cultures are made which are likewise incubated at 37 degrees. If any colonies showing the characteristic appearance of the cholera spirillum are found, these are examined microscopically, and if comma-shaped organisms are present, inoculations are made into fresh tubes to be further tested by means of the indol reaction and by inoculation into animals.

Stokes and Hachtel (1912) have suggested the use of a modified Hesse agar containing starch for the isolation of the cholera spirilla, which produce acid on such a medium, while the colon-typhoid organisms do not. The intestinal spirilla as a class form round, spreading, pinkish colonies on the starch medium, while colonies of other intestinal bacilli remain blue. The medium is best used after the Koch enrichment method described above.

Other pathogenic organisms have been isolated from waters,

according to the accounts of numerous investigators, but from the sanitary point of view the typhoid and cholera bacilli are of most importance, since these are manifestly the germs of disease most likely to be disseminated through this medium. For the detection of *Bact. anthracis* and other spore-forming pathogenic bacteria which may at times gain access to water from stockyards, slaughter-houses, etc., the method suggested by Frankland (Frankland, 1894) may be adopted. The water to be examined is heated to 90 degrees for 2 minutes and then plated, the characteristic colonies of the anthrax organism being much more easily discerned after the destruction of the numerous non-sporing water bacteria.

Brown, Petroff and Heise (1916) by examining large volumes of water were able to isolate tubercle bacilli from the stream which receives the sewage from Saranac Lake at points as far as three and a half miles from the sewer outlet.

The Hydrogen Sulphide Test for Fæcal Pollution. Chamot and Redfield (Redfield, 1912; Chamot and Redfield, 1915) have suggested a new test for fæcal organisms in water, based on the production of hydrogen sulphide in a simple pepton medium by the proteolytic organisms. The method is interesting and the results reported indicate that very pure waters usually fail to show hydrogen sulphide production after 72 hours' incubation, while contaminated waters give positive results in 12–24 hours. The test is positive with many samples which do not show *Bact. coli* and it seems to the authors probable that it would condemn many waters of satisfactory sanitary quality.

CHAPTER VIII

THE SIGNIFICANCE AND APPLICABILITY OF THE BACTERIOLOGICAL EXAMINATION

Sanitary Inspection and Sanitary Analysis. The first attempt of the expert called in to pronounce upon the character of a potable water should be to make a thorough sanitary inspection of the pond, stream, well or spring from which it is derived. Study of the possible sources of pollution on a watershed, of the direction and velocity of currents above and below ground, of the character of soil and the liability to contamination by surface-wash are of supreme importance in interpreting the analyses to be made. In many parts of the country protests arose during the early years of the use of the Treasury Department Standard against too literal and rigid an application of any arbitrary analytical standards (Morse and Wolman, 1918; Hinman, 1920). In the first Progress Report of the Commission appointed by the Treasury Department to Recommend Standards of Purity for Drinking Water supplied to the Public by Common Carriers Engaged in Interstate Traffic (Reprint No. 232, U. S. P. H. S.) it was stated that "It is a fact so well established as to need no further discussion that the results of bacteriological and chemical examination of a sample of water ought always to be correlated with the knowledge of the source, treatment and storage of the supply in order to enable a just estimate of the sanitary quality of such supply." The Advisory Committee is at present engaged in the fuller formulation of an official statement in regard to the importance of the Field Survey as a factor in estimating the sanitary quality of water with a detailed presentation of the chief elements which should enter into such a survey. This report when it is published will be found invaluable as a guide to all who are engaged in sanitary surveys of the sources of potable water.

The field survey and the laboratory analysis are mutually complementary. Whittaker (1917) reports that out of 344 water supplies found to be polluted in Minnesota 52 per cent were

condemned on the ground both of field survey and laboratory analysis; 40 per cent were condemned from the survey although the analysis on the date of examination was satisfactory; in 8 per cent of the cases the danger was revealed only by analysis, the first survey indicating nothing suspicious. If house or barnyard drainage or sewage is actually seen to enter a water used for drinking purposes it is obviously unnecessary to carry out delicate chemical or bacteriological tests to detect pollution. On the other hand, no reconnoissance can show certainly whether unpurified drainage from a cesspool does or does not reach a given well; whether sewage discharged into a lake does or does not find its way to a neighboring intake; whether pollution of a stream has or has not been removed by a certain period of flow. Evidence upon these points must be obtained from a careful study of the characteristics of the water in question, and this study can be carried out along two lines, chemical and bacteriological.

Sanitary Chemical Analysis. A chemical examination of water for sanitary purposes is mainly useful in throwing light upon one point — the amount of decomposing organic matter present. It also gives an historical picture which may be of much value. Humus-like substances may be abundant in surface-waters quite free from harmful pollution, but these are stable compounds. Easily decomposable bodies, on the other hand, must obviously have been recently introduced into the water and mark a transitional state. " The state of change is the state of danger," as Dr. T. M. Drown once phrased it. Sometimes the organic matter has been washed in by rain from the surface of the ground, sometimes it has been introduced in the more concentrated form of sewage. In any case, it is a warning of possible pollution, and the determination of free ammonia, nitrites, carbonaceous matter, as shown by " oxygen consumed," and dissolved oxygen yield important evidence as to the sanitary quality of a water.

Furthermore, nitrates, the final products of the oxidation of organic matter, and the chlorine introduced as common salt into all water which has been in contact with the wastes of human life, furnish additional information as to the antecedents of a sample. The results of the chlorine determination are indeed perhaps more clear than those of any other part of the analysis, for chlorine and sewage pollution vary together, due allowance

being made for the proximity of the sea and other geological and meteorological factors. Unfortunately, it is only past history and not present conditions which these latter tests reveal, for in a ground-water completely purified from a sanitary standpoint such soluble constituents remain, of course, unchanged. Thus, in the last resort, it is upon the presence and amount of decomposing organic matter in the water that the opinion of the chemist must be based.

Information Furnished by Bacteriological Examinations. The decomposition of organic matter may be measured either by the material decomposed or by the number of organisms engaged in carrying out the process of decomposition. The latter method has the advantage of far greater delicacy, since the bacteria respond by enormous multiplication to very slight increases in their food-supply, and thus it comes about that the standard gelatin-plate count at 20° roughly corresponds, in not too heavily polluted waters, to the free ammonia and "oxygen consumed," as revealed by chemical analysis. If low numbers of bacteria are found, the evidence is highly reassuring, for it is seldom that water could be contaminated under natural conditions without the direct addition of foreign bacteria. The bacteriologist in such cases can declare the innocence of the water with justifiable certainty. When high numbers are found, the interpretation is less simple, since they may exceptionally be due to the multiplication of certain peculiar water forms. Large counts, however, under ordinary conditions, when including a normal variety of forms, indicate the presence of an excess of organic matter, derived in all probability either from sewage or from the fresh washings of the surface of the ground. In either case danger is indicated.

A still closer measure of polluting material may be obtained from the numbers of colonies which develop on litmus-lactose-agar at 37°, since organisms which thrive at the body temperature, and particularly those which ferment lactose, are characteristic of the intestinal tract and occur but rarely in normal waters.

Finally, the search for *Bact. coli* furnishes the most satisfactory of all single tests for fæcal contamination. This organism is preeminently a denizen of the alimentary canal and may be isolated with ease from waters to which even a small proportion of sewage has been added. On the other hand, it is never found in abundance in waters of good sanitary quality, and its numbers form an

excellent index of the value of waters of an intermediate grade. The full bacteriological analysis should then consist of three parts, the gelatin-plate count, as an estimate of the amount of organic decomposition in process; the total count, and the count of red colonies, on litmus-lactose-agar, as a measure of the organisms which form acids and thrive at the body temperature; and the study of a series of lactose broth tubes for the isolation of colon bacilli.

Special Advantage of the Bacteriological Examination. The results of the bacteriological examination have, in several respects, a peculiar and unique significance. First, this examination is the most *direct* method of sanitary water analysis. The occurrence of nitrites or free ammonia in a small fraction of one part per million, or of chlorine in several parts per million, do not in themselves render a water objectionable or dangerous. They merely serve as indicators to show that germ-containing and germ-sustaining organic matter is present. By a determination of the chlorine and study of the relations of carbon and nitrogen, it is possible to determine with some degree of accuracy whether this organic matter is of plant or animal origin, and hence to rate its objectionable or dangerous character. *By the bacteriological examination, on the other hand, we are able to determine directly whether particular kinds of organisms characteristic of sewage are, or are not, actually present in the water.* What we dread in drinking-water is the presence of pathogenic bacteria, mainly from the intestinal tract of man, and it is quite certain that the related non-pathogenic bacteria from the same source will behave more nearly as these disease germs do than will any chemical compounds. In the second place, the bacteriological methods are superior in *delicacy* to any others. Klein and Houston (1898) showed by experiment with dilutions of sewage that the colon test was from ten to one hundred times as sensitive as the methods of chemical analysis; and studies of the self-purification of streams have confirmed their results on a practical scale. Thus in the Sudbury River it was found that while chemical evidences of pollution persisted for 6 miles beyond the point of entrance, the bacteria introduced could be detected for 4 miles further (Woodman, Winslow, and Hansen, 1902).

The statement is sometimes made that while bacteriological methods may be more delicate for the detection of pollution in

surface-waters, contamination in ground-waters may best be discovered by the chemical analysis. That such is not the case has been well shown by Whipple (Whipple, 1903) who cites the following two instances in which the presumptive test revealed contamination not shown by the chemical analysis:

"A certain driven-well station was located in swampy land along the shores of a stream, and the tops of the wells were so placed that they were occasionally flooded at times of high water. The water in the stream was objectionable from the sanitary standpoint. The wells themselves were more than 100 feet deep; they penetrated a clay bed and yielded what may be termed artesian water. Tests for the presence of *Bact. coli* had invariably given negative results, as might be naturally expected. Suddenly, however, the tests became positive and so continued for several days. On investigation it was found that some of the wells had been taken up to be cleaned, and that the workmen in resinking them had used the water of the brook for washing them down. This allowed some of the brook-water to enter the system. It was also found that at the same time the water in the brook had been high, and because of the lack of packing in certain joints at the top of the wells the brook-water leaked into the suction main. The remedy was obvious and was immediately applied, after which the tests for *Bact. coli* once more became negative. During all this time the chemical analysis of the water was not sufficiently abnormal to attract attention. On another occasion a water-supply taken from a small pond fed by springs, and which was practically a large open well, began to give positive tests for *Bact. coli*, and on examination it was found that a gate which kept out the water of a brook which had been formerly connected with the pond was open at the bottom, although it was supposed to have been shut, thus admitting a contaminated surface-water to the supply." Whipple also calls attention to the report on the Chemical and Bacteriological Examination of Chichester Well-waters by Houston (Houston, 1901), in which the results of chemical and bacteriological examinations of thirty wells were compared. It was found that the bacteriological results were in general concordant and satisfactory. The wells which were highest in the number of bacteria showed also the greatest amount of pollution, as indicated by the numbers of *Bact. coli*, *Bact. sporogenes*, and streptococci. On the other hand, the chlorine and the

albuminoid ammonia showed no correspondence with the bacteriological results.

Vincent (Vincent, 1905) cites an interesting case of the detection of progressive pollution of a ground-water by bacteriological methods. The well of a military camp in Algeria showed 200 bacteria per c.c. before the arrival of a regiment of troops. Its subsequent history is indicated in the table below:

PROGRESSIVE POLLUTION OF A WELL
(VINCENT, 1905)

	Bacteria per c.c.	Bact. coli per c.c.
Before arrival of troops	200	0
6 days after arrival	770	0
14 days after arrival	4,240	1
41 days after arrival	6,960	2
60 days after arrival	14,900	10

Thirdly, negative tests for *Bact. coli* and low bacterial counts may be interpreted as proofs of the good quality of water, with a *certainty* not attainable by any other method of analysis. Many a surface-water with reasonably low chlorine and ammonias has caused epidemics of typhoid fever; but it is impossible, under any natural conditions (except perhaps in a well polluted with urine) that a water could contain the typhoid bacillus without giving clear evidence of pollution in the fermentation tube or on the lactose-agar plate.

In the examination of springs, especially those used for domestic supplies at country houses, the authors have found that the bacteriological examination offers a more delicate and more certain index of the quality than may be obtained by chemical analysis. In a number of instances, springs located in pastures have become slightly polluted by animals, but to so small an extent that the chemical examination gave no indication of trouble. The bacteria, however, increased greatly in number, and colon bacilli could be readily isolated from 75 per cent of the 1-c.c. samples of a long series used in making the presumptive test. A single case may suffice as an illustration. This was a spring located on a hill in Hopkinton, Mass.

The chemical analysis was as follows:

Color	None
Turbidity	None
Sediment	None
Odor (hot)	None
Odor (cold)	None
Total solids	33.0000
Loss on ignition	7.0000
Fixed residue	26.0000
Hardness	11.0000
Chlorine	10.0000
Nitrogen as —	
Albuminoid ammonia	0.0000
Free ammonia	0.0000
Nitrites	0.0000
Nitrates	0.0000

The bacteriological examination showed a total count of 375 bacteria per c.c. and a 37° count of 350 per c.c. The presumptive tests for *Bact. coli* showed that gas-producing organisms were present in a majority of 1-c.c. samples, and typical colon bacilli were isolated. In this case the contamination was brought about by cattle gaining access to the area immediately surrounding the spring; but the same conditions might easily have led to infection from human beings.

Fromme (1910) cites several interesting examples of temporary pollution detectable only by bacteriological tests. The most striking case was that of an artesian well. Its average bacterial content had been 38 per c.c. and colon bacilli were absent from 200 c.c. In May, 1908, this well became polluted from a broken stable drain 10 meters away. The number of bacteria rose to 4370 and colon bacilli were found in 10 c.c. samples. The source of pollution was removed, but the well water in July still contained 7100 bacteria and *Bact. coli* in 1 c.c. In September the number had fallen to 105 and colon bacilli were present in 200 c.c. In November the bacteria numbered 120 and colon bacilli were absent from 200 c.c. At no time did chemical tests give any indication of danger, while the bacteriological data obviously measured very delicately a comparatively slight but real pollution and its gradual disappearance.

A special case, in which the bacteriological examination will prove of special value is in the study of bottled waters which are

always liable to contamination in handling, a type of contamination which could in general be revealed only by bacteriological methods. Various investigators, whose work is reviewed by Koser and Skinner (1922), report high counts and the presence of colon bacilli in soft drinks, and the systematic examination of such products which are so commonly exposed to various possibilities of hand contamination should receive the serious attention of health authorities.

It seems to the writers that the real application of chemistry begins where that of bacteriology ends. When pollution is so gross that its existence is obvious and only its amount needs to be determined, the bacteriological tests will not serve, on account of their excessive delicacy. In studying the heavy pollution of small streams, the treatment of trades wastes, and the purification of sewage, the relations of nitrogenous compounds and of oxygen compounds are of prime importance. In other words, when pollution is to be avoided, because the decomposition of chemical substances causes a nuisance, it must be studied by chemical methods. When the danger is sanitary and comes only from the presence of bacteria, bacteriological methods furnish the best index of pollution.

In the study of certain special problems the paramount importance of bacteriology is generally recognized. The distribution of sewage in large bodies of water into which it has been discharged may thus best be traced on account of the ready response of the bacterial counts to slight proportions of sewage, particularly since the ease and rapidity with which the technique of plating can be carried out make it possible to examine a large series of samples with a minimum of time and trouble. The course of the sewage carried out by the tide from the outlet of the South Metropolitan District of Boston was studied in this way by E. P. Osgood in 1897, and mapped out by its high bacterial content with greater accuracy than could be attained by any other method. Some very remarkable facts have been developed by similar studies as to the persistence of separate streams of water in immediate contact with each other. Heider showed that the sewage of Vienna, after its discharge into the Danube River, flowed along the right bank of the stream, preserving its own bacterial characteristics and not mixing perfectly with the water of the river for a distance of more than 24 miles (Heider, 1893). Jordan

(Jordan, 1900), in studying the self-purification of the sewage discharged from the great Chicago drainage canal, found by bacteriological analyses that the Des Plaines and the Kankakee Rivers could both be distinguished flowing along in the bed of the Illinois, the two streams being in contact, yet each maintaining its own individuality. Finally, the quickness with which slight changes in the character of a water are marked by fluctuations in bacterial numbers renders the bacteriological methods invaluable for the daily supervision of surface supplies or of the effluents from municipal filtration plants.

In the commoner case, when normal values obtained by such routine analyses are not at hand, the problem of the interpretation of any sanitary analysis is a more difficult one. The conditions which surround a source of water supply may be constantly changing. No engineer can measure the flow of a stream in July and deduce the amount of water which will pass in February; yet the July gauging has its own value and significance, so a single analysis of any sort is not sufficient for all past and future time. If it gives a correct picture of the hygienic condition of the water at the moment of examination it has fulfilled its task, and this the bacteriological analysis can do. The evidence furnished by inspection and by chemical analysis should be sought for and welcomed whenever it can be obtained, yet we are of the opinion that, on account of their directness, their delicacy, and their certainty, the bacteriological methods should never be omitted.

The Development of Water Laboratories. It may be of interest to note the remarkable growth of water laboratories in recent years as evidence of the extent to which the importance of the subject is now being realized. This development has been particularly rapid since 1910. Thus of 83 city water laboratories listed by Hinman (1918) 3 were established before 1900, 23 between 1900 and 1910 and 60 between 1910 and 1917. A resumé of the situation in 1920 based on the survey of the Committee on Municipal Health Department Practice of the American Public Health Association was presented by Professor I. V. Hiscock of the Yale Medical School before the American Chemical Society at its April, 1923, meeting. Practically every one of the 83 cities of 100,000 population and over which were studied has provided for systematic laboratory control of its water supply.

In a group of 60 of these cities furnishing data in regard to the work of laboratories devoted particularly to water supply control 44 make daily bacteriological examinations of the treated water while in 7 cities such examinations are made several times a day and in 9 cities less often than once a day. In addition to the work of these water laboratories, the general laboratories of the health department generally conduct routine examinations of the public water supply. In a group of 68 such cities 44,939 bacteriological examinations of water were made giving an average of nearly 13 per city per week.

The bacteriological results obtained in 24 cities reporting fully in regard to this point are cited in the table below and furnish interesting evidence of the high quality of the vast majority of the supplies in question.

CHARACTER OF THE WATER SUPPLIED TO 24 LARGE CITIES OF THE UNITED STATES IN 1920. (Hiscock)

Number of cities in each class.

	Per cent of Samples Examined Showing Bact. Coli in	1 c.c.				10 c.c.			
Source		Lake	River	Combination	Total	Lake	River	Combination	Total
Raw water	Less than 50%	4	5	0	9	1	4	0	5
	50-75%	1	2	1	4	1	1	0	2
	Over 75%	0	10	1	11	3	12	2	17
Treated water	Less than 1%	3	15	1	19	2	8	0	10
	1-2%	1	2	1	4	1	1	0	2
	Over 2%	1	0	0	1	2	8	2	12
Total cities		5	17	2	24	5	17	2	24

CHAPTER IX

BACTERIOLOGY OF SEWAGE AND SEWAGE EFFLUENTS

Bacteriological and Chemical Examination of Sewage. The first object of modern sewage disposal is the oxidation of putrescible organic matter. Chemical, rather than bacterial, purification is usually the prime requisite; and chemical tests therefore serve best as criteria of the results obtained. Bacteria are the agents in the process of sewage purification; but the most generally useful measure of the work accomplished is the chemical oxidation attained. "To employ a simile, it is a case of the saw and the 2-foot rule — the saw will do the cutting, but the rule will measure the work cut." (W. J. Dibdin.)

In certain cases, however, bacterial as well as chemical purity must be effected, in view of special local requirements. The sewage from a contagious disease hospital, for example, should be freed from infectious material as a factor of safety. Sewage discharged into a body of water adapted for bathing should be so treated as to protect those using the water. In the case of seaboard cities where sewage effluents are likely to contaminate oyster beds and other layings of edible shellfish the problem assumes great importance. Where bacterially impure effluents are discharged into streams used for sources of water-supply the town taking water may protect itself by filtration. It should so protect itself, in any case, from the pollution necessarily incident to surface waters; and, unless the bacterial condition of a stream or lake is made materially worse by the discharge of sewage effluents, it is fair that the responsibility of purification should rest on the water works, rather than on the sewage purification plant. The whole question is one of relative costs. Under certain circumstances sanitary authorities may rightly demand that bacteria, as well as putrescible organic matter, shall be removed in sewage treatment. Under such conditions the bacterial control of purification plants is as essential as in the case of water filters.

Methods of Bacteriological Examination of Sewage and Effluents. In England, considerable attention has been devoted to this subject, and numerous methods have been recommended as

furnishing valuable criteria of the bacterial quality of sewage effluents. Houston (1902[b]), for example, suggests various tests involving the use of litmus milk, peptone solution, gelatin tubes, and neutral-red broth, as well as the inoculation of animals. He considers the determination of the numbers of *Bact. coli* and *Bact. sporogenes* as of greatest moment, while the identification of streptococci is of value in certain cases and the enumeration of liquefying bacteria, spore-forming aerobes, thermophilic bacteria, and hydrogen sulphide producing bacteria is of subsidiary importance. Rideal (1906) subsequently recommended a somewhat less extensive series of tests, including aerobic and anaerobic counts, both at 20 and 37°, with the determination of the number of liquefiers and the number of spore-formers. The results attained do not seem to warrant any such elaborate procedure. As far as the authors are aware, the determination of liquefying bacteria, anaerobic bacteria and thermophilic bacteria does not add any information of material importance to that obtained from the total count. Some test for specific sewage organisms is of course desirable. Here again, however, the determination of *Bact. sporogenes* and sewage streptococci tells the observer little more than can be learned from the routine use of the colon test. In the United States the practise of sewage bacteriologists is crystallizing around the total count and the estimation of *Bact. coli*. In the absence of evidence as to the specific value of other data, the routine control of filter plants may well be limited to these two determinations.

The total count of bacteria should be made, as in the case of waters, at 20°. Determinations carried out in duplicate at 37° give additional information of considerable value. The ratio of the 37° count to the 20° count varies with different sewages. At Boston the body temperature count is 70 to 80 per cent of the total count; at Lawrence it appears to be proportionately much lower (Gage, 1906). In using either medium, it is well to add lactose and litmus and note the number of red colonies, as a check on the enumeration of *Bact. coli*.

It should be borne in mind, as Lederer and Bachmann (1911) have pointed out, that the sampling error is a very serious one with sewage. Duplicate tests made at 1-minute intervals for a period of 10 minutes in their experiments gave extreme values of 190,000 and 550,000 per c.c.

The determination of the number of colon bacilli in sewage and effluents should furnish an integral part of bacteriological sewage analysis, since it is important to know whether the decrease of intestinal bacteria in the process of purification is proportional to the reduction of total bacteria. The State Sewerage Commission of New Jersey has adopted this procedure in its supervision of the disposal plants in that State; and the results seem amply commensurate with the labor involved. As in the case of polluted waters the enumeration of *Bact. coli* may be carried out, either by the study of the red colonies which appear on litmus-lactose-agar plates inoculated with the sample directly, or by the use of a preliminary enrichment process. The complete identification of *Bact. coli* seems unnecessarily tedious, however, where the organisms are present in such abundance. Some approximate presumptive method is indicated here, if anywhere; and the experience with polluted water, reviewed in Chapter VI, points to the Jackson bile medium as the most promising one. Experience at the Sewage Experiment Station of the Massachusetts Institute of Technology has shown that this presumptive test in general yields good results. As pointed out above, a 48-hour incubation period at 37° is required. All tubes showing 20 per cent gas at the end of this time may be considered positive tests for the colon group, without serious error.

The Committee on Sewage Works Operation (1915) of the American Public Health Association recommends presumptive tests only (either gas production or the formation of typical red colonies on litmus lactose agar plates) for the control of the operation of sewage treatment plants.

Numbers of Bacteria in Sewage. The total number of bacteria and the number of colon bacilli naturally vary widely in the sewages of different cities and towns. European sewages, being more concentrated, show as a rule higher numbers than are found in America. Results compiled from various sources show from 1,000,000 to 5,000,000 bacteria in the sewages of Essen, Berlin, Charlottenburg, Leeds, Exeter, Chorley, and Oxford; 2,000,000 to 10,000,000 in the sewages of London, Walton, and W. Derby; and over 10,000,000 in the sewages of Paris, Ballater and Belfast (Winslow, 1905). The number of colon bacilli in English sewages varies from 50,000 to 750,000. In American sewages, on the other hand, bacteria are somewhat less numerous. At Lawrence

the determinations made from 1894 to 1901 showed on the average 2,800,000 bacteria per c.c. At Worcester, Eddy reported 3,712,000 in 1901 (Eddy, 1902); at Ames, Iowa, Walker (1901) found 1,248,256 in the same year. At Columbus, Johnson (1905) reports an average of 3,600,000 bacteria per c.c.; the individual numbers varied from 320,000 to 27,000,000. The number of colon bacilli varied from 50,000 to 1,000,000 and averaged 500,000. Day samples of Boston sewage collected three times a week, from October, 1906, to April, 1907, showed an average of 1,200,000 bacteria per c.c. In the summer months numbers are notably higher than at other seasons in many sewages. Thus in 1903, Boston sewage contained 2,995,000 bacteria in July, 4,263,600 in August, 11,487,500 in September, 3,693,000 in October, 587,100 in November, and 712,000 in December (Winslow, 1905). There is also a marked diurnal variation in the bacterial content of sewage, since the flow contains a smaller proportion of intestinal matter at night than at other times. For example, a series of hourly samples at the Sewage Experiment Station of the Massachusetts Institute of Technology showed the following results:

BACTERIA IN BOSTON SEWAGE — AVERAGES FOR EACH FOUR-HOUR PERIOD. AUGUST 13–14, 1903

(Winslow and Phelps, 1905)

Period	Bacteria per c.c.
7:30–11:30 A.M.	1,800,000
11:30 A.M.–3:30 P.M.	3,200,000
3:30–7:30 P.M.	4,600,000
7:30–11:30 P.M.	3,500,000
11:30 P.M.–3:30 A.M.	1,000,000
3:30–7:30 A.M.	400,000

It is evident that many published results of bacterial examinations of sewage are in excess of the average values, since they refer in most cases to day samples only.

Where certain industrial wastes are present abnormally low bacterial counts may be found in municipal sewage. Thus at New Haven (Winslow and Mohlman, 1918) a test carried out for one week at intervals during the day time gave the following results:

BACTERIAL CONTENT OF NEW HAVEN SEWAGE

	Agar 20° Count per c.c.				Gas Formers per c.c.			
	8 A.M.	10 A.M.	1 P.M.	4 P.M.	8 A.M.	10 A.M.	1 P.M.	4 P.M.
Average 6 week days	1,295,000	115,400	14,700	159,000	100,000	19,000	385	17,000
Sunday	.....	3,355,000	2,275,000	2,535,000	....	100,000	100,000	100,000

At 8 a.m. and on Sundays the bacterial content was reasonably normal; but during the working day the 20° count was kept down to less than 200,000 and the gas formers to less than 20,000 per c.c. by copper salts introduced from a munition plant.

Bacterial Content of Sewage Effluents. The bacterial content of sewage effluents varies widely according to the process of purification adopted and the efficiency of the particular plant. Of the ordinary treatment processes adopted, activated sludge treatment and intermittent sand filtration are the only ones which produce a notably purified effluent from a bacteriological standpoint.

The activated sludge process may be made to yield almost any bacteriological results desired by adjusting the aeration and sedimentation periods, the amount of air and the proportion of activated sludge. This is well illustrated by the results tabulated below from the Milwaukee experiments.

RELATION BETWEEN AERATION TIME AND BACTERIAL PURIFICATION

(Milwaukee, 1915)

Aeration period, hours.....	0	1	2	3	4	5
Per cent removal of bacteria.....	0	52	81	92	95	98

In actual practice very high degrees of purification are not usually attained. Thus at the permanent Milwaukee plant the monthly average 20° count varied in a recent period from 714,000 to 1,761,000 and the activated sludge effluent from 35,000 to 190,000.

Intermittent sand filters, when operated with care, may give a bacterial purification well over 99 per cent as shown by bacteriological examinations at the Brockton (Mass.) filters, reported by Kinnicutt, Winslow and Pratt (1910) as follows:

BACTERIA IN SEWAGE AND EFFLUENTS AT BROCKTON, AVERAGE OF FOUR EXAMINATIONS, AUTUMN OF 1908

	Bacteria per c.c. Gelatin 20°	Colon Bacilli per c.c. Lactose Bile
Sewage	3,150,000	150,000
Effluent A	1,900	400
" B	6,300	15
" D	125	0
" E	1,400	5
" F	2,000	1

Such high efficiencies as this table indicates are often not realized under the actual working conditions of a municipal plant. At Vineland, N. J., for example, the intermittent filters show a reduction of 90 to 95 per cent in total bacteria and a somewhat higher reduction of *Bact. coli*. The results of three examinations made in 1906 are given below.

BACTERIA IN SEWAGE AND SAND FILTER EFFLUENT AT VINELAND, N. J.

(N. J. State Sewerage Commission, 1907)

Date	Bacteria per c.c		Bact. Coli in	
	Sewage	Effluent	Sewage	Effluent
March 2	480,000	20,000	0.0001 c.c.	0.01 c.c.
July 26	496,000	61,000	0.0001 c.c.	0.001 c.c.
July 26	511,000	38,000	0.00001 c.c.	0.001 c.c.

Contact beds, and trickling filters naturally show a much less satisfactory bacterial removal than sand filtration beds. In the Columbus experiments, Johnson (1905) found from 1,000,000 to 2,000,000 bacteria in the effluents of contact beds and from 750,000 to 1,900,000 in the effluent from trickling filters.

At the experiment station of La Madeleine, in Lille, Calmette (1907), reports 5,000,000 bacteria per c.c. in the crude sewage, 2,900,000 in the second contact effluent and 800,000 in the effluent from the trickling bed. Of 20,000 *Bact. coli* per c.c. applied to the filters, the contact system delivered 4000 and the trickling bed 2000 per c.c. The average results of examinations made three times a week at the Sewage Experiment Station of the Massachusetts Institute of Technology, during two different periods, were as follows:

BACTERIA IN SEWAGE, SEPTIC EFFLUENT AND TRICKLING EFFLUENT AT BOSTON

(Winslow and Phelps, 1907)

	Bacteria per c.c.				Bact. Coli Positive Tests in 0.000001c.c.*
	July–Sept., 1906		Oct., 1906–April, 1907		July–Sept., 1906
	No.	Per cent Reduction	No.	Per cent Reduction	Per Cent
Sewage	1,300,000	.....	1,200,000	.....	65
Septic effluent	1,650,000	Inc.	750,000	38	66
Effluent from trickling bed	750,000	42	200,000	83	35
Septic tank and trickling bed	750,000	42	180,000	85	35

* Jackson bile test.

BACTERIAL CONTENT OF SEWAGE AND EFFLUENTS FROM TRICKLING FILTERS

Place	Period	Bacteria per c.c.		
		Screened Sewage	Septic Effluent	Filter Effluent
Reading, Pa.	1908–1909	3,100,000	1,800,000	600,000
Columbus, Ohio	1909	2,370,000	1,050,000	560,000

The preceding average data for two of the largest trickling filter plants in the United States are cited by Kinnicutt, Winslow and Pratt (1910).

It is obvious that effluents of this character cannot be considered satisfactory from the standpoint of bacterial purification. As Houston concluded, after a careful review of the subject, " The different kinds of bacteria and their relative abundance appear to be very much the same in the effluents as in the crude sewage. Thus, as regards undesirable bacteria, the effluents frequently contain nearly as many *Bact. coli*, proteus-like germs, spores of *Bact. enteritidis-sporogenes* and streptococci, as crude sewage. In no case, seemingly, has the reduction of these objectionable bacteria been so marked as to be very material from the point of view of the epidemiologist" (Houston, 1902[a]).

Experimental studies with specific bacteria have confirmed these conclusions. Houston (1904[b]) found that *Ps. pyocyanea* appeared in the effluent of a trickling bed 10 minutes after application to the top and continued to be discharged for 10 days. In septic tanks and contact beds, the same germ persisted for 10 days. Rideal (1906) quotes experiments by Pickard at Exeter, which show that typhoid bacilli may persist for 2 weeks in a septic tank and that contact bed treatment effects only a 90 per cent removal of these organisms.

Disinfection of Sewage Effluents. Where bacterial purity is required, some special process of disinfection must be combined with the contact bed or the trickling filter. For this purpose treatment with chloride of lime or liquid chlorine is generally used; and in connection with this process bacteriological control is an essential.

Rideal (1906) first showed at Guildford that 30 parts of available chlorine per million would reduce the number of bacteria in crude sewage from several millions to 50,000, while 50 parts would reduce their number to 20 per c.c. Colon bacilli were reduced from one million per c.c. to less than one per c.c. by 30 parts of chlorine. In septic effluent 25 to 44 parts of chlorine per million reduced *Bact. coli* from two and a half to four and a half million per c.c. to less than one per c.c. With contact effluents smaller amounts of chlorine proved efficient. The primary effluent required 20 parts per million, the secondary effluent 10.6 parts per million and the tertiary effluent 2.5 parts per million to reduce the

number of *Bact. coli* so that this organism could not be isolated in 5 c.c.

In this country Phelps and Carpenter (1906) demonstrated the practical usefulness of bleaching powder disinfection, at the Sewage Experiment Station of the Massachusetts Institute of Technology. As indicated in the table below smaller amounts of chlorine than were used by Rideal will give good results with more dilute American sewages.

BACTERIA IN TRICKLING FILTER EFFLUENT BEFORE AND AFTER TREATMENT WITH CHLORIDE OF LIME (5 PARTS PER MILLION AVAILABLE CHLORINE)

(PHELPS AND CARPENTER, 1906)

Date	Bacteria per c.c.		Bact. coli, Jackson Bile Test	
	Before	After	Before 0.000001 c.c.	After 1.0 c.c.
1906				
August 11.....	270,000	69	+ 0	+ 0
" 13.....	630,000	41	0 0	+ 0
" 14.....	135,000	406	+ +	+ 0
" 15.....	230,000	21	0 0	0 0
" 16.....	250,000	37	+ 0	0 0
" 18.....	110,000	40	0 0	+ 0
" 20.....	90,000	54	+ 0	0 0
" 21.....	220,000	22	0 0	0 0
" 23.....	.....	.....	+ 0	0 0
Average.....	240,000	86	33%+	22%+
Average removal.....	99.96%		99.993%	

The success of chemical disinfection varies with the character of the sewage or effluent treated, since the organic matter present consumes a certain amount of the disinfectant and renders it inoperative. Discordant results are therefore reported from different sources.

An important series of experiments carried out in Ohio by Kellerman, Pratt, and Kimberly (1907) showed good results with sand filter effluents and contact effluents. Septic sewage, on the other hand, required large amounts of chlorine to produce a

reasonable bacterial reduction. The table herewith shows the results obtained at Marion, Ohio.

BACTERIA IN SEPTIC EFFLUENT, CONTACT EFFLUENT, AND SAND EFFLUENT AT MARION, O., BEFORE AND AFTER TREATMENT WITH CALCIUM HYPOCHLORITE

(KELLERMAN, PRATT, AND KIMBERLY, 1907)

Date	Effluent	Average Available Chlorine Parts per Million	Bacteria per c.c.			
			20° C.		37° C. Total Count	
			Untreated	Treated	Untreated	Treated
1907						
Apr. 11	Septic	4.3	850,000	1,100,000	1,200,000	240,000
Apr. 12	Septic	6.2	4,400,000	550,000	850,000	260,000
Apr. 15	Septic	7.6	600,000	400,000	450,000	190,000
Apr. 28	Contact	2.9	110,000	2,500	.....	.....
Apr. 29	Contact	5.0	65,000	1,600	73,000	370
Apr. 30	Contact	4.4	500,000	800	160,000	400
Mar. 21	Sand	3.8	49,000	570	9,800	150
Mar. 22	Sand	3.0	56,000	140	7,000	60
Mar. 26	Sand	1.5	70,000	4,000	20,000	160

Date	Effluent	Average Available Chlorine Parts per Million	Bacteria per c.c.			
			37° C. Red Colonies		Bact. coli	
			Untreated	Treated	Untreated	Treated
1907						
Apr. 11	Septic	4.3	55,000	7,400		
Apr. 12	Septic	6.2	60,000	15,000		
Apr. 15	Septic	7.6	100,000	51,000		
Apr. 28	Contact	2.9	.....	.....	20,000	Not in 0.5
Apr. 29	Contact	5.0	10,000	0	15,000	" 0.5
Apr. 30	Contact	4.4	21,000	3	20,000	" 1.0
Mar. 21	Sand	3.8	1,300	0	1,000	" 1.0
Mar. 22	Sand	3.0	800	0	2,000	" 1.0
Mar. 26	Sand	1.5	4,000	1	2,000	In 1.0

In Germany, on the other hand, Schumacher (1905), Kranepuhl (1907), and Kurpjuweit (1907) found larger amounts of chlorine necessary, in the neighborhood of 60 parts per million

parts of sewage. Their tests were somewhat severe, however, the criterion of success being the absence of *Bact. coli* in a large proportion of liter samples.

The bacterial results which may be attained under conditions where an extremely high degree of purification is required are well illustrated by the plant at Mt. Kisco, N. Y., where the sewage from this village is treated successively by septic tanks, contact beds, sedimentation, sand filters and chlorination before it enters a tributary of the New York water supply.

BACTERIAL PURIFICATION, MT. KISCO
(COFFIN AND HALE, 1916)

	Sewage	Septic Effluent	First Contact Effluent	Second Contact Effluent	Settled Effluent	Sand Effluent	Chlorinated Effluent
Bacteria per c.c. (37° agar)	1,480,000	400,000	560,000	470,000	460,000	38,000	38
Bact. coli per cent .001 c.c. samples positive	37	22	20	16	14	0	0

The final chlorinated effluent showed *Bact. coli* in only 39 per cent of a series of 10 c.c. samples and in only 8 per cent of a series of 1 c.c. samples.

Standards for Sewage Effluents. The science of sewage bacteriology is still in an unadvanced state. We know that bacterial counts will vary with the degree and type of purification but it is difficult to give any general rules for the interpretation of bacteriological examinations designed to indicate whether disposal plants are operating successfully or not. Houston stated provisionally in 1902 that the 20° count should be under 100,000 and the 37° count under 10,000, while *Bact. coli* should be absent from .001 c.c. and *Bact. sporogenes* from .1 c.c. This standard now seems to us far too lenient, especially in view of the great improvements which have been made in methods of treatment. It seems wisest at the present time to avoid fixing any definite standards of purity for sewage effluents. Each case should be judged intelligently on its own merits. In general, however, where bacterial purification is indicated at all, it seems fair to

demand that the effluent should be of such a quality as not materially to increase the bacterial content of the body of water into which it is discharged.

Bacteriology of the Sewage Filters Themselves. Before leaving the subject of sewage bacteriology, brief reference must be made to the importance of bacteriological studies in relation to the processes of sewage purification which bring about the removal of the organic matter itself. Notwithstanding the great advances in the development of the art of sewage disposal, knowledge of the micro-organisms concerned and of the exact conditions which favor their activity is greatly to be desired; but such knowledge is woefully deficient. In a general way we know of the nitrifying organisms long ago discovered by Winogradsky. More recent work, like that of Schultz-Schultzenstein (1903), Boullanger and Massol (1903) and Calmette (1905), has slightly increased our knowledge concerning these forms; but there is no clear cut statement as to the part played by these organisms. In regard to the specific bacteria functioning in the septic tank and contact bed we are almost wholly in the dark. Present opinion seems to indicate that certain specific types of bacteria play a predominant part. Septic tanks work well with some sewages and badly with others; and the presence or absence of the right bacteria is probably largely responsible for the different results. In some cases, as at Plainfield, N. J., the seeding of a tank with cesspool contents has produced a material improvement in septic action. The process of inoculation or seeding is now not uncommon, e. g., in Imhoff tanks. The fact that a filter works best when "ripe" suggests the presence of specific organisms with rather definite growth and reacting conditions.

Knowledge of the kinds of bacteria involved would make it possible to substitute scientific control for such empiricism and might well lead to improved methods of a more intensive character than are yet available. The work already done upon a laboratory scale furnishes promise of such results. The student who wishes to follow out this line of investigation will find a good summary of fundamental data in regard to the hydrolysis and cellulose and other carbohydrates in Rideal's "Sewage and the Bacterial Purification of Sewage" (1906), and additional facts are scattered through numerous recent reports and proceedings, but definite knowledge of the important types is still lacking.

Gage (1905) has made a suggestive study of the bacteria which

carry on the reducing changes in sewage which deserves the attention of all who are interested in the more theoretical aspects of sewage treatment. His method consisted in plating sewages and effluents, isolating typical cultures and determining their power to decompose peptone and nitrates with the production of ammonia and free nitrogen. The rate of gelatin liquefaction, the amount of nitrate reduced, the amount of free ammonia formed, and the amount of nitrogen liberated were quantitatively determined for each culture thus isolated. The numerical values obtained, multiplied by the number of bacteria, apparently of the same type, observed in the plates, gave coefficients of the liquefying, denitrifying, ammonifying, and nitrogen-liberating power of the effluent; and these coefficients may be considered as measures for a given sample of the tendency of the bacterial flora to set up certain changes. The results of further studies made by Clark and Gage (1905), on sewages and on sand, contact, and trickling effluents, show that there may be important differences between various sewages in this respect which must render their purification more or less easy. They indicate that the effluents obtained from intermittent sand filters in cold weather contain larger numbers of ammonifying and denitrifying bacteria than appear at other seasons, which may help to explain the poorly nitrified effluents obtained in the winter season.

A highly promising series of investigations along this line is now being conducted at New Brunswick, New Jersey, through the coöperation of the Agricultural Experiment Station and the Department of Health of the State. A preliminary communication by Hotchkiss and Murray (American Journal of Public Health, July, 1923) presents valuable and suggestive information in regard to the distribution of various groups of bacteria in the Imhoff tank.

Birge (1915) studied the effects of certain common aerobic bacteria grown alone and together in sterilized sewage and demonstrated the reducing power of *Bact. coli* and *Bact. cloacae* and the ammonifying power of *Bact. subtilis* (under aerobic condition) and of Proteus forms (under anaerobic conditions). Buswell and Long (1923) have more recently presented some interesting results in regard to the biological agents which function in activated sludge, with particular reference to the higher fungi and protozoa. Research work in the micro-biology of sewage treatment, along such lines as these, promises to be highly fruitful of results.

CHAPTER X

BACTERIOLOGICAL EXAMINATION OF SHELLFISH

Shellfish and Disease. The pollution of areas devoted to the growing of shellfish and the consequent pollution of the shellfish themselves is a matter of much sanitary importance. Oysters, clams and mussels are the shellfish commonly used as food, and since they are likely to be eaten in an uncooked or partially cooked condition, it is important to be assured as to their character from the bacteriological standpoint. In their normal habitats, in clean sea-water, or in river estuaries free from pollution, shellfish are unquestionably free from dangerous bacteria, although their feeding habits make it probable that the types of bacteria indigenous to the waters in which they are found might be present in considerable numbers. With the pollution of streams by unpurified sewage the areas in which oysters and clams develop may easily become infected by organisms of intestinal types, and there is, therefore, offered an easy means for the typhoid bacillus and other pathogenes to pass from the sewage directly into the intestinal tract of the consumer of the raw oysters or clams.

The history of this subject is well summarized by Newlands and Ham (1910), from whose excellent report the following paragraphs are adapted:

Attention was first drawn to the danger from shellfish by the remarkable outbreak of typhoid fever which occurred in Middletown, Conn., in 1894, as a result of the serving of raw oysters at college fraternity banquets. The oysters used in this case were all derived from a certain portion of Long Island Sound, where they had been put down, or planted, in order to fatten. Investigation showed that the stream entering the Sound at this point was highly polluted, and furthermore, that at a nearby house there were two severe cases of typhoid fever from which the intestinal discharges were turned into the drain and thence into the stream without disinfection. The course of the passage of the bacteria from the patient suffering with the disease to the oyster and so on to the young men at the banquets was, therefore,

traced out in a most complete and thorough way. This investigation, which was conducted by Prof. H. W. Conn, of Wesleyan University, caused immediate investigations to be set on foot in this country.

In 1893 Thorne-Thorne, in a report to the Local Government Board, wrote that, in his opinion, certain cases of cholera which had occurred that year at various inland towns in England were due to eating contaminated oysters from beds at Grimsby, where there had been a small cholera epidemic. Following the suggestions embodied in this report the English Government began a series of investigations which have made many important additions to our present knowledge of the subject.

In 1902 the famous oyster epidemics at Winchester and Southampton, England, were proven beyond reasonable doubt to have been caused by contaminated oysters taken from grounds at Emsworth. Here again we have to deal with banquets given in different cities where the only common source of infection appears to have been contaminated oysters. Of the 267 guests at these banquets 118 were attacked with intestinal disorders and 21 cases of typhoid fever developed, 5 of which were fatal.

Although a great many sensational attacks, based on insufficient or no evidence, have been made against oysters as carriers of disease germs, the above-mentioned investigations and others, among which might be mentioned those of Thresh, Marvel, and Soper, have brought out sufficient trustworthy evidence to show that contaminated oysters must be considered as a real factor in the dissemination of typhoid fever and other water-borne diseases. An estimate of the exact extent to which such illness is due to oysters would be impossible. The careful supervision exercised by health authorities over oyster culture during recent years has certainly greatly minimized the danger. In practically all the American epidemics which have been clearly traced to shellfish it was demonstrated that the oysters which caused the outbreak had been "floated" or "fattened" in brackish water near the mouths of polluted streams; and this practice is no longer permitted by sanitary authorities.

Valuable studies of the relation between shellfish and disease have been published by Bulstrode (1911) and Wilhelmi (1911) and Stiles (1912).

Effect of Cookery upon Polluted Shellfish. It should be noted that it is unfortunately not only raw shellfish which are responsible for the spread of disease. Most of the processes of cookery to which these foods are subjected are insufficient to destroy pathogenic germs. Clark (1906) found that clams and oysters in stews and fried and scalloped in the usual manner were generally free from colon bacilli and streptococci. With steamed clams, however, the bacteria present could not be destroyed except by a temperature high enough and prolonged enough to ruin the clams for eating. Rickards (1907) confirmed these results as to the danger from steamed clams, while he found fried clams and clams in chowder and scalloped oysters to be practically sterilized. Oyster stew, however, is not exposed to long continued heat as is clam chowder, and fried oysters are less thoroughly heated than fried clams in the ordinary processes in use. Oysters in both of these forms and fancy roast oysters still contained colon bacilli and streptococci. Buchan (1910) finds that the ordinary methods of cooking mussels do not remove the risk of typhoid infection.

Bacteriological Examination of Shellfish. Without further discussing the general sanitary aspects of the subject it is important to consider just how one may determine whether the oysters from a given region are polluted or not. The methods which have been developed for this work are essentially modifications of the methods used in water examination, involving sometimes total counts of bacteria at different temperatures, but especially the application of the various tests for the determination of the colon bacillus, since here, as in water examination, this organism may be taken as an index of pollution and its occurrence in considerable numbers must be looked upon not merely with suspicion, but as a practical proof that the supernatant waters are polluted and that the shellfish themselves may contain organisms of pathogenic importance, such as *Bact. typhosum*, *Bact. dysenteriæ*, *Bact. sporogenes* and others. Determinations of the pollution of the water above the beds are sometimes made as bearing indirectly and inferentially on the possibility of the pollution of the shellfish contained therein. Results of the two determinations are not always in close agreement, however, owing to the rapidly changing local conditions due to tide, etc. The general relations and the individual variations between water and shellfish determinations

BACTERIA IN WATER AND SHELLFISH, NEW HAVEN HARBOR

Station	Water				Oysters		
	Samples Taken	Av. Number Bacteria per c.c.		Average Number Bact. coli* per c.c.	Average Number Bact. coli* per c.c.	Number Oyster Samples	Character of Bottom
		37° C.	20° C.				
Ferry St. Bridge	12	210	1260	43	.....	.....	Soft
Tomlinson Bridge	15	910	2650	34	.....	.....	"
No. 1	15	510	1680	51	.....	.....	"
No. 2	15	375	910	73	.....	.....	"
No. 3	16	255	835	9	72	16	"
Buoy 10	15	155	450	10	.....	.....	"
No. 4	15	160	1720	9	.....	.....	"
No. 5	17	615	1340	74	308	13	"
Buoy 5	23	315	715	15	.....	.....	"
Buoy 8	15	205	410	8	.....	.....	"
No. 6	16	145	485	8	37	6	Seaweed
No. 7	21	215	740	29	425	11	Hard
No. 9B	11	220	260	7	64	10	"
No. 9A	13	100	185	9	46	10	"
No. 9	12	195	200	17	37	10	"
No. 7A	11	120	240	10	255	11	"
No. 8	16	180	270	7	370	6	
No. 10	23	300	615	9	100	1	Soft
No. 11	11	405	510	8	10	4	"
Buoy 6	21	815	1690	9	.....	.....	
Buoy 3	17	175	590	6	291	8	Hard
No. 12	14	620	1190	4	6	5	"
No. 13	7	240	120	10	10	8	"
No. 14	12	285	1100	1—	7	8	"
Buoy 4	7	375	1400	4			
No. 15	7	455	1680	1	45	15	Hard
No. 16	14	280	1025	1—	1—	3	Soft
No. 17	14	300	1260	1—	.....	.....	Hard
No. 19	1	800	300				
No. 20	10	135	860	1—	10	8	Hard
Buoy 2	11	375	905	2			
No. 22	8	305	560	1—	7.3	3	Hard
Buoy 1	6	115	995	1—	4	12	"
No. 18	10	255	675	1—			
No. 24	4	710	1340	4	9	10	Hard
No. 23	6	450	240	1—			"
No. 25	2	130	1000	1—			
No. 26	5	130	465	1—			
No. 27	10	630	695	1—	.....	.....	Soft
No. 28	4	415	1400	1—	4	3	Mud and sand
No. 29	5	370	1700	1—	.....	.....	Soft
No. 30	5	185	440	1—	1—	15	Hard
No. 31	7	320	130	1—	1—	15	"
No. 32	5	70	1050	1—	3	15	"
No. 33	4	485	405	1—	1—	10	"
No. 34	4	535	495	1—	1	10	"
No. 35	4	120	270	1—	1	15	Sticky

* Jackson's lactose bile presumptive test used.

Minus sign after figure 1 indicates that the average was less than 1.

are well illustrated in the table on page 162 from the report by Newlands and Ham (1910) on conditions in New Haven Harbor.

Study of the methods of examination of shellfish has been conducted with great care at the Lawrence Experiment Station by Gage, at the Sanitary Research Laboratory at the Institute of Technology by Phelps, at Brown University by Gorham, and in New York by Pease. Other officials of the Shellfish Commissions of different States have also carried out investigations upon this subject.

It has been noted that the superiority of lactose bile to dextrose broth is greatest in water examinations when the water is most polluted. In the study of shellfish the danger of overgrowths is even greater than in polluted waters, since the organic matter in the oyster and its surrounding shell water furnishes a culture medium for many bacteria. Streptococci are particularly abundant. As pointed out in Chapter VII, streptococci die out more rapidly than colon bacilli in potable waters, but where organic matter is present in abundance the former may survive the latter.

We have compiled the table given below from the results of Clark (1906). In all cases except in that of the shell water there is a considerable difference between the dextrose fermentation tests and the colon isolations, indicating an overgrowth by streptococci and other forms, of colon bacilli originally present. The *Bact. sporogenes* is also very frequently responsible for such anomalous results in shellfish examinations.

COLON BACILLI AND STREPTOCOCCI IN DIFFERENT PORTIONS OF THIRTY CLAMS

	Per Cent of Samples Showing			
	Fermentation in Dextrose Broth	Bact. coli	Streptococci	Bact. coli and Streptococci
Shell water	90	83	47	40
Gills	77	53	25	15
Stomach (intestine)	55	35	22	12
Rectum (intestine)	82	45	43	13
Liver	37	18	15	3
Visceral tissue	18	8	7	2

It will be noted from Clark's table above that the shell liquor is not only freer from overgrowths than the portions of the body of the clam, but that the proportion of positive reactions is in each case higher. Since the shell water is of course easier to examine than the macerated animal, this is now generally adopted as the standard material for examination.

It should be noted, however, that the feeding habits of the oyster may explain many of the variations in numbers of bacteria in oysters from the same source.

Since 1910 the importance of the problems involved in the bacteriological examination of shellfish has been recognized by the maintenance of a committee of the Laboratory Section of the American Public Health Association on Standard Methods for the Examination of Shellfish. The first report of this committee was made in 1910 and the most recent report (Committee on Standard Methods for the Examination of Shellfish, published in Journal American Public Health Association, July, 1922) was made in 1921, and is as follows:

Oysters in the Shell

"*Collection of Sample.* — At least twelve oysters of the average size of the lot under examination, with deep bowls, short lips and shells tightly closed, shall be selected and prepared for transportation to the laboratory.

As complete a record of such data as it is possible to obtain shall be made, covering the following points: the exact location of the bed from which the samples have been collected, the depth of water over the bed at time of collection, the state of the tide, the direction and velocity of the wind, other weather conditions, the day and hour of removal of stock from the water, the conditions under which the stock has been kept prior to the taking of the sample, provided that the sample was not taken for laboratory testing directly from the water, and the conditions under which sample was kept between time of collection and of examination.

Transportation of Sample. — The oysters so selected shall be placed in a cloth bag or other suitable container, which shall be marked for identification. If the oysters are analyzed the same day as collected no icing is necessary. If kept for a longer period, either in transit or in the laboratory before examination, they shall be iced in such a manner as to prevent mixing the ice water

with the oysters. At all times they should be kept in as cool a place as is convenient.

Condition of Sample. — The analyst shall make a record of the general condition of the oysters before the analysis of the sample, as to whether the shells are open or closed, and as to the presence of any abnormal odors. No oysters from which the shell liquor has escaped shall be used for examination.

Technical Procedure. — The bacteriological examination shall be started as soon as possible after the receipt of the sample. The oysters shall be thoroughly cleansed by scrubbing with a stiff brush and clean running water, and dried. The edges of the shell shall be sterilized by passing through a flame or by burning with alcohol. The oysters shall be opened by an oyster knife which has been sterilized by flaming. The shell liquor of at least five oysters shall be collected aseptically in a sterile wide-mouth bottle, the oysters being drained until the shell liquor ceases to run away freely.

Five 1 c.c. portions of the composite sample of shell liquor obtained by draining from five to twelve oysters, and five 1 c.c. portions of 0.1 and 0.01 dilutions of the composite liquor shall be placed in fermentation tubes containing standard lactose broth. The water used for dilution purposes shall be either sterile sea water or sterile tap water containing 2 per cent sodium chloride.

The fermentation tubes shall be incubated for two days at 37° C. Upon the formation of gas confirmatory tests shall be made in accordance with the standard methods of water analysis. From the fermentation tubes showing gas transfers shall be made on Endo or litmus lactose agar plates. Characteristic types of *Bact. coli* colonies shall then be transferred to a second lactose broth fermentation tube and to a slant agar tube. From the agar slant a microscopic examination shall be made to show the organism in a non-spore forming bacillus.

Bacterial Counts. — It is recommended that the determination of the total number of bacteria present in the shell liquor be discontinued.

Oysters Removed from the Shell

(Opened or Shucked Stock)

Collection of Sample. — The stock in the container from which the sample is to be taken shall be thoroughly mixed by shaking and stirring, and at least one-half pint of the sample placed in a sterile wide-mouthed jar by means of a ladle or other implement sterilized by flaming, alcohol being usually the most convenient material for use in the field. The jar shall then be tightly stoppered.

Transportation of Sample. — When the time between collection of the sample and its examination shall exceed three hours, or if the outside temperature is above 50° F., the sample shall be kept cool by means of ice placed around, but not in the sample jar.

Technical Procedure. — Two hundred c.c. of sterile 2 per cent salt solution shall be placed in a sterile container and oyster meats added sufficient to bring the water level up to the 400 c.c. graduation mark on the container. A stoppered Erlenmeyer flask, glass jar, or other container which can be easily sterilized, and on which the graduation may be plainly marked, may be used.

The contents of the container shall be thoroughly shaken and the watery fluid used for the determination of *Bact. coli* as detailed for the examination of the shell liquor of oysters in the shell.

As the oyster meats are diluted with an equal quantity of saline solution the result shall be multiplied by two for a score.

Expression of Results

Shell and Shucked Stock

The results of the bacteriological examination for *Bact. coli* shall be expressed by the following arbitrary numerical system, known as the American Public Health Association Method of Scoring Oysters.

If desired, the higher dilutions may be run for shucked oysters and for shell stock. In this case a 0.001 dilution has a positive value of 1,000 and a 0.0001 dilution of 10,000 and so on.

The presence of *Bact. coli* in each fermentation tube, if confirmed, is to be given the following values, which represent the reciprocals of the greatest dilutions in which the test for *Bact. coli* is positive:

If present in 1.0 c.c. but not in 0.1 c.c. the value of 1.

If present in 0.1 c.c. but not in 0.01 c.c. the value of 10.

If present in 0.01 c.c. the value of 100.

The addition of these values for the five fermentation tubes gives the total value for the sample and this figure is the score, the tube representing the greatest dilution for each set being counted.

The results are expressed in the following tabular form:

RESULTS OF TESTS FOR BACT. COLI IN DILUTIONS INDICATED

Fermentation Tubes	1.0 c.c.	0.1 c.c.	0.01 c.c.	Numerical Value
1	*	*	0	10
2	*	*	0	10
3	*	0	0	1
4	*	0	0	1
5	*	0	0	1
				Score: 23

* Positive, confirmed *Bact. Coli* in fermentation tube.
0 Negative, *Bact. Coli* absent.

Sometimes results similar to the following are obtained; that is, one or more tubes may show positive results in small quantities of shell water while other tubes may show negative results in larger quantities. In this case a recession of values is made and the next lower numerical value is given to the positive results in the high dilution and such positive result is considered as being transferred to a lower dilution giving a negative result in another set of tubes.

As examples of the method of obtaining the score, the following tables are given:

RESULTS OF BACT. COLI TESTS IN DILUTIONS INDICATED

Tubes	1.0 c.c.	0.1 c.c.	0.01 c.c.	Value
1	*	*	0	10
2	*	*	0	10
3	*	*	0	10
4	*	0	0	10
5	*	*	*	10
				Score: 50

Tubes	1.0 c.c.	0.1 c.c.	0.01 c.c.	Value
1	*	*	0	10
2	*	*	0	10
3	*	*	*	100
4	*	*	*	10
5	*	0	0	10

Score: 140"

Seasonal Variation of Bacteria in Oysters. It has been observed by Gorham (1912) and others that the examination of oysters from certain regions made in the summer fail to agree with the similar analyses from the same beds made in the winter. With the advent of cold weather there seems to be a great improvement in the sanitary quality, so that oysters taken from beds in close proximity to the outfalls of large sewers show in the colder months entire absence of any evidence of contamination, judged solely by the bacteriological data. Thus Gorham found in the summer of 1910 that all oysters on the beds in the Providence and Warren Rivers and the upper part of Narragansett Bay were so badly polluted by sewage as to be unfit for food. Colon bacilli were found in the "shell water" of every oyster in amounts as small as .01 of a cubic centimeter or less. Chemical and bacteriological examination of the waters over these beds showed them to be heavily sewage polluted. In December of the same year the analyses of the oysters were strikingly different, although the condition of the water was apparently unchanged. In the examination five oysters were selected in each case and the average total number of bacteria per cubic centimeter was determined and the presence of colon bacilli was tested by the bile tube and subsequent isolation and identification of the organisms. The table on page 169 shows the numbers of bacteria found, and the proportion of the five oyster samples in which colon bacilli were present in cubic centimeter amounts and also in 0.1 and 0.01 of a cubic centimeter.

The conclusions arrived at by Gorham are that during the cold weather the oysters assume a condition of rest or hibernation, during which time ciliary movement ceases and the process of feeding is suspended. No organisms are therefore taken in from the outside water and those inside the oyster are gradually eliminated, so that the total number of organisms is reduced very

considerably and the oyster becomes practically free from colon bacilli.

SEASONAL VARIATION IN THE BACTERIAL CONTENT OF OYSTERS
(GORHAM, 1912)

Date	Average Total Bacterial Count of Shell Water of Five Oysters.	Proportion of Five Oysters Showing Bact. coli in			Score	Bact. coli Present in Water in	Temperature of Water C.
		1 c.c.	0.1 c.c.	0.01 c.c			
BED NO. 8. PROVIDENCE RIVER							
Dec. 20, 1910.....	1000	3	1	0	4	0.01 c.c.	−1°
Jan. 14, 1911.....	750	5	3	1	41		
Jan. 25.....	80	4	3	0	23	0.01 c.c.	1°
Jan. 27.....	23	5	3	0	32		
Feb. 10.....	130	2	2	0	4	1.0 c.c.	0.1°
Feb. 28.....	140	0	0	0	0	0.0001 c.c.	1°
Mar. 11.....	200	5	4	0	41	0.01 c.c.	1.75°
April 14.....	275	5	2	0	23	0.01 c.c.	8.5 °
April 28.....	700	5	5	4	410	0.0001 c.c.	12.5°
May 12.....	1700	5	5	5	500	0.0001 c.c.	15°
BED NO. 44. PROVIDENCE RIVER							
Jan. 7, 1911.....	425	5	5	1	140	.....	0.25°
Feb. 10.....	250	4	0	0	4	.....	0°
Feb. 28.....	240	5	1	0	14	.....	0.5°
March 11.....	100	5	2	0	23	.....	2°
April 14.....	210	2	0	0	2	.....	8.5°
April 28.....	1000	5	5	4	410	.....	11.75°
May 12.....	1100	5	5	4	410	.....	14.75°
BED NO. 204. WARREN RIVER							
Jan. 25, 1911.....	600	5	4	1	50	.....	0°
Feb. 10.....	140	0	0	0	0	1.0 c.c.	0°
Feb. 28.....	400	0	0	0	0	0.01 c.c.	0.75°
March 4.....	750	3*	3*	0*	*	.....	0.75°
March 11.....	60	1	0	0	1	0.01 c.c.	3°
March 14.....	3400	0	0	0	0	0.01 c.c.	8.75°
April 28.....	1050	5	5	4	410	0.01 c.c.	13°
BED NO. 205. WARREN RIVER							
Dec. 22, 1910.....	250	3	0	0	3	.....	−1°
Feb. 10, 1911.....	325	0	0	0	0	1.0 c.c.	0°
Feb. 28.....	450	4	2	0	14	0.01 c.c.	1°
March 4.....	600	2	2	1	5	.....	0.75°
March 11.....	85	2	1	0	3	0.01 c.c.	2°
April 14.....	325	1	1	0	2	0.01 c.c.	8.25°
April 28.....	4000	5	5	5	500	0.01 c.c.	11.5°

* Only three oysters used.

Self-purification of Shellfish. In connection with the bacteriological examination of shellfish for colon bacilli certain investigations have been carried out which are of great importance from the commercial as well as from the sanitary standpoint. Phelps (1911) has shown that oysters which develop in waters subject to sewage pollution may be purified or entirely freed from colon bacilli by the removal of the oysters themselves to waters of purer character, when, after sufficient time has elapsed, the oysters will have cleansed themselves through their metabolic processes and become entirely safe even for consumption in the raw state. It is of considerable importance to determine the length of time necessary for this self-purification to take place. Obviously, from the commercial standpoint it is desirable to make it as short as possible, while from the sanitary standpoint it must be long enough to insure a thorough and satisfactory removal of all traces of polluted matter. Oyster beds which are free from pollution or which are sufficiently good for the re-laying for polluted oysters are difficult to find and limited in areas because of their nearness to sources of pollution. The investigations in question were conducted by Phelps in the Providence River and the upper part of Narragansett Bay. The oysters were removed from heavily polluted regions and carried to waters which were practically free from pollution, where they were planted. Examinations were made from day to day in order to determine the length of time that these particular oysters showed pollution and it was found that within 4 days the organisms of the colon type were practically all eliminated.

The purification of oysters has been extensively developed on a practical scale by Wells (1916), the essential principle of the process being the storage of the oysters in a special basin containing chlorinated water. A more recent contribution by the same author (Wells, 1923) describes the first purification plant of this type to be formally recognized by a certificate from the conservation commission of the state of New York. It includes two concrete basins with a capacity of 6000 gallons each and each capable of handling 200 bushels of oysters. The chlorine for disinfecting is obtained by electrolysis of sea water. The oysters are exposed for two successive periods of respectively 6 and 12 hours to the action of the chlorinated water with the result that initial scores of 50–500 are reduced by the first "drinking interval" to 5–50 and by the second treatment to scores of 0–5.

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